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Expression of biologically active human corticosteroid-binding globulin and analysis of the steroid binding site by site-directed mutagenesis and chemical modification

Ghose-Dastidar, Jayasri, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

EXPRESSION OF BIOLOGICALLY ACTIVE HUMAN CORTICOSTEROID BINDING GLOBULIN AND ANALYSIS OF THE STEROID BINDING SITE BY SITE-DIRECTED MUTAGENESIS AND CHEMICAL MODIFICATION

by

JAYASRI GHOSE-DASTIDAR

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5/12/92
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Executive Officer

Lesley Davenport

Ronald A. Kohanski

Jay C. Unkeless

Supervisory Committee

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Abstract

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by

Jayasri Ghose-Dastidar

Advisers: Professor J. B. Alexander Ross

Professor Reza Green

Human corticosteroid binding globulin (hCBG) is a serum glycoprotein that binds the steroid hormones cortisol and progesterone with high affinity. It appears that hCBG may have important physiological functions, such as delivery of steroid to sites of inflammation. To understand the biogenesis of hCBG and to analyse its steroid binding site, the cDNA sequence has been isolated, and expressed in the baculovirus/insect cell system. Quantitation of the expressed hCBG secreted by these cells shows that high levels of biologically active recombinant hCBG can be produced in this system ($\approx$50 pmol/2.5X10^6 cells derived medium). Thus this system should be useful for producing sufficient hCBG for physico-chemical analysis. Further characterization of this recombinant hCBG shows that, although the secreted hCBG is high mannose containing, it has identical affinities and specificities for different

steroids as that of native hCBG. This indicates that processing of core-glycosylated hCBG to complex type carbohydrate containing species in the Golgi compartment is not necessary for hCBG to achieve a steroid binding conformation. Instead, the binding properties of unglycosylated hCBG produced in insect cells, demonstrate that the process of N-linked glycosylation is crucial for hCBG to attain its biologically active form. However, N-glycosylation is not the sole determinant of the active conformation, since the *in vitro* translated, N-glycosylated hCBG does not bind cortisol. It thus appears that intracellular N-glycosylated hCBG undergoes some further structural maturation during intracellular transport.

To address the possible role of specific cysteine residues in the steroid binding process (as suggested by previous studies), mutant hCBGs containing serine or alanine in place of cys_{228}, have been produced by site-directed muatgenesis and construction of corresponding recombinant baculoviruses. Scatchard analysis shows that alteration of cys_{228} to serine or alanine does not change the steroid binding affinity of hCBG, demonstrating that cys_{228} is not involved in the binding interaction. This finding leads to the hypothesis that cys_{60} may be the functionally important cysteine. By modifying the wild-type and mutant hCBGs with the sulfhydryl-specific reagents N-ethylmaleimide (NEM), iodoacetamide (IAA), and sodium tetrathionate, it has been demonstrated that cys_{60} is present at the steroid binding site and may possibly be involved in the steroid binding process. Requirement of dithiothreitol treatment for achieving complete inactivation of binding activity by NEM and sodium tetrathionate strongly indicates that cys60 is the accessible cysteine which is partially oxidized under native conditions.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The present work attempts to understand the biogenesis of human corticosteroid binding globulin (hCBG) in the cellular secretory pathway and to identify the biochemical and biophysical parameters involved in the steroid binding activity of this protein. In a broader sense, this work addresses the general issue of the mechanism of protein folding. Since hCBG is a glycoprotein of moderate molecular weight and monomeric structure, and has very high affinities for the steroid hormones cortisol and progesterone, it appears to be a good model system for studying general structure-function relationships in protein.

First, I planned to establish a system for biological expression of hCBG in which hCBG could be produced at a high level. Second, using this system, I aimed to investigate the role of glycosylation in the biogenesis of hCBG. For a variety of glycoproteins, diversified roles for glycan moieties in intracellular transport and in biological activity have been suggested by several workers (Reviewed in Olden et al. 1982). Since hCBG is a glycoprotein, it was of interest to examine whether glycosylation of hCBG plays any role in the secretion and function of this protein.

By combining site-directed mutagenesis with chemical modifications, I aimed to investigate whether specific cysteine residues are involved in the steroid binding site of hCBG, as suggested by others (Khan and Rosner, 1977; Hammond et al., 1987). Presently, site-directed mutagenesis is widely used to elucidate structure-function relationship of proteins. The unique feature of this method is that it allows one to introduce a desired change in a local environment of a protein, and ask whether that change brings any alteration in function. By introducing conservative or

nonconservative amino acid changes, one can predictively maintain or destroy the physical character of a local environment. A combined approach of site-directed mutagenesis and chemical modification has further allowed me to dissect the roles of two cysteine residues in the steroid binding of hCBG.

Historical aspects

The classical concept of steroid hormone action involves binding to, and activation of, intracellular receptors, which in turn modulate at the transcriptional level the expression of different genes. This genomic mode of steroid hormone action is well studied and understood. How steroid molecules enter cells, however, remains unclear, although it is generally believed that they cross the cell membrane by passive diffusion. The presence of several high affinity binding proteins specific for steroid hormones in the extracellular circulation makes the picture complicated. These proteins are less abundant than serum albumin, which binds steroids loosely, but are responsible for binding ≥80% of circulating steroid molecules. The investigation of the interaction between steroids and their binding proteins became more important in the context of how steroid molecules are targeted to the cell membrane. It has long been controversial whether steroid binding proteins have a more complex role in steroid function beyond protecting them from degradation and maintaining appropriate serum levels.

It has been pointed out by Westphal (1986) that probably it is an oversimplification that the unbound fraction of steroids in serum is the sole determinant of bioactivity. Under physiological conditions, there is always a dynamic

equilibrium between a steroid molecule and its high affinity binding protein that determines the availability of the biologically active steroid hormones entering target cells. Steroid binding proteins thet have been characterized physically and biochemically include sex hormone binding globulin (SHBG) (Petra, 1991), corticosteroid binding globulin (CBG) (Hammond, 1990), vitamin D binding protein (DBP) (Cook et al., 1991).

Human CBG (hCBG) is a serum glycoprotein of molecular weight 52 KD (Kato et al., 1988) that is mainly synthesized in the liver, and is secreted into the circulation. The presence of CBG in human serum was first discovered by Daughaday (1956) and by Bush (1957) independently. CBG is ubiquitous in almost every vertebrate species (Seal and Doe, 1963). Even in a lower eucaryotic fungus like *Candida albicans*, a unique corticosteroid binding protein is identified which is more similar to serum CBG than the intracellular glucocorticoid receptor (Loose et al., 1981; Loose and Feldman, 1982).

Although hCBG is known to be synthesized in liver, CBG-like molecules have been found in many other cell types. CBG has been differentiated from intracellular glucocorticoid and progesterone receptors by its inability to bind the synthetic glucocorticoid dexamethasone and the synthetic progesterone R5020. For example, in pituitary, more than one type of glucocorticoid-binding molecule was found, and one of these was found to have properties identical to serum CBG (Koch et al.,1976). It was further suggested that this intracellular CBG may control the extent of interaction between GR and glucocorticoids. It is not clear whether this cellular CBG is due to uptake from serum or is synthesized *in situ* (Kloet et al.,1977).

CBG-like molecules were also found in lung (Giannopoulos, 1976), muscle (Mayer et al.,1975), lymphocytes (Werthamer et al., 1973), thymocytes (Schaumburg et al.,1968), and uterus (Milgrom and Baulieu, 1970; Al-Khouri and Greenstein, 1980). By using immunocytochemical techniques, the presence of rat CBG immunoreactivity has been further demonstrated within cells of liver, kidney, anterior pituitary, thyroid, and uterus (Kuhn et al., 1986). Also, in guinea pig, the specific presence of CBG has been demonstrated in pituitary corticotrophs (Perrot-Applanat et al.1984).

Physical properties and molecular structure

The sequence of hCBG cDNA (1423 bp) predicts that hCBG is a 405 amino acid long polypeptide (Hammond et al.,1987) with a 22 amino acid amino-terminal hydrophobic sequence, not present in mature hCBG, which is predicted as the signal peptide. hCBG contains six consensus sequences for Asn-linked glycosylation (Asn-X-Ser/Thr where X is not proline/ (Montreuil & Vliegenthart, 1979), out of which five sites are actually utilized (Akhrem et al., 1982). The hCBG gene is composed of five exons distributed over approximately 19 Kb (Underhill and Hammond, 1989.). Sequence comparison indicates that the organization of hCBG gene is most closely related to α-1-proteinase inhibitor and α-1-antichymotrypsin genes, suggesting that these proteins are derived from a common ancestral gene. By hybridization of the radiolabeled cDNA with metaphase chromosomes, it has been further shown that hCBG gene is located in q31-q32.1 region of chromosome 14 (Seralini et al., 1990). Notably, the genes for α-1-proteinase inhibitor and α-1-antichymotrypsin are also located in this region, supporting the idea that these

proteins arose from gene duplication events during evolution.

hCBG specifically binds gluocorticoids and progesterone with very high affinities, with the highest affinity for cortisol. It is important to emphasize that hCBG is highly distinct from intracellular glucocorticoid receptor (GR) in that it has no sequence homology with GR (Hollenberg et al., 1985), and it does not bind dexamethasone, a widely used synthetic glucocorticoid analog; however, both of these macromolecules are specific binders of natural glucocorticoids. As such the major question about hCBG is what kind of structural design at the steroid binding site dictates its steroid binding specificities, and how this might differ within hCBG and GR.

Endocrine regulation of hCBG and cortisol

The mechanisms that regulate the levels of hCBG and cortisol under different physiological conditions, appear to be complex and possibly, interrelated. It has been observed that treatment with dexamethasone decreases the production of CBG (Frairia et al., 1988), whereas adrenalectomy increases the circulating level of hCBG (Kawai & Kuzuza, 1972). It is also well established that estrogen raises serum CBG levels (as found in pregnant women and subjects treated with estrogens). However, under these conditions, there is also parallel rise in total and free cortisol in plasma. It is not known why the increased cortisol level does not downregulate the CBG level in plasma under these conditions. Another relevant question concerns the physiological stimulus for CBG synthesis and secretion. In the sheep fetus, a rise in cortisol late in gestation is associated with a rise in CBG concentration in fetal

plasma (Ballard et al., 1982). Challis et al. (1985) reported that the rise in fetal plasma CBG is stimulated by ACTH administration. Also, they found that this increase in CBG is positively correlated with the increase in plasma cortisol. Jacobs et al. (1991) investigated the source of the increased level of fetal plasma CBG and the effect of ACTH treatment on CBG mRNA. They have reported that the increase in fetal plasma CBG is due to increased CBG mRNA synthesis (mainly in liver and in some other tissues), and is not derived from transplacental transfer or placental synthesis. Furthermore, they have shown that ACTH treatment stimulates biosynthesis of CBG in fetal kidney. Ali and Basset (1991) later reported that a specific CBG-like protein of adrenal origin is released along with corticosterone upon stimulation of adrenal slices with ACTH.

It is still unclear if cortisol acts directly on hepatocytes to influence the biogenesis and secretion of CBG. In case of retinol binding protein (RBP), it has been observed that retinol can induce the secretion of RBP from rat hepatocytes (Ronne et al., 1983)). Since, in a retinol-deficient cell line, RBP accumulates in the rough endoplasmic reticulum (RER), the authors concluded that the transport of RBP from RER to the Golgi is regulated by retinol. A similar situation may arise in case of CBG. Possibly, cortisol may affect the tertiary structure of CBG during its biogenesis and/or it may be a stimulus for CBG secretion. In this respect, the successful expression of hCBG in insect cells should help understand this aspect of hCBG biogenesis. Since these cells do not have any endogeneous CBG-like molecules, and they lack cortisol (most insects synthesize ecdysone as their major steroid), this system allows one to investigate the effects of specific ligands in the

biogenesis of hCBG and its secretion.

Another unexplained phenomenon is the rise of cortisol itself during pregnancy and in subjects treated with estrogens (Clerico et al., 1978; Angeli et al., 1977; Smith et al.,1980) Although it is known that, in both of the above conditions, progesterone, which is another ligand for hCBG, also increases in serum, the high increase in cortisol can not be explained by replacement of cortisol from hCBG by progesterone (Doe et al, 1969). They suggest that the rise in free plasma cortisol occurs as a result of estrogen administration, and that this increase is dose-related. On the other hand, it is also known that cortisol controls its own synthesis by regulating the release of ACTH from pituitary through a negative-feedback mechanism. An increase in the free cortisol level would be expected to inhibit ACTH secretion, and thus reduce adrenal output until the free cortisol level has returned to normal. Since this appears not to be the case, an alternative hypothesis has been suggested, that CBG and estrogen may reset the level at which the pituitary or hypothalamus is inhibited by free cortisol (Doe et al., 1969).

Possible functions of hCBG

The sequence of hCBG cDNA shows striking homology to all members of the family of serine protease inhibitors, specially to α-1-antitrypsin (Hammond et al.,1987). These enzymes share the property that they are cleaved by their target enzymes. It was found that hCBG is cleaved by neutrophil elastase, a serine protease which is released at sites of inflammation (Pemberton et al., 1988). The resulting shorter hCBG polypeptide has a lower affinity for cortisol. The authors hypothesized

that this proteolytic cleavage of CBG may result in the specific release of bound cortisol. In a later investigation, Hammond et al. (1990) demonstrated that when hCBG was incubated with either leukocyte elastase or granulocytes from a septic patient, it was cleaved to a smaller molecular weight form which had very low binding activity. Taken together, these studies suggest that hCBG may have some role in delivering cortisol to sites of inflammation.

Endo and Fuzihira (1990) reported that granuloma cell cytosol contains at least two cortisol binding proteins. By comparing the properties of the granuloma binding protein to the serum CBG in terms of its ligand binding specificities, heat stability of ligand binding and ammonium sulfate fractionation, they found that this cytosolic protein closely resembles serum CBG.

The presence of CBG-like molecules in various glucocorticoid target organs raises the possibility that CBG may be involved in specific transport of glucocorticoids across the cell membrane (Kuhn, 1988; Singer et al.,1988). Furthermore, the presence of specific binding sites for CBG on target cell membranes has been reported (Kuhn, 1988) which bind CBG with an approximate K_d of μM, the concentration at which CBG is present in serum. The hypothesis that CBG may have some role in steroid uptake is further supported by the study of Hryb et al (1986) who reported specific binding sites for hCBG on membranes prepared from human prostate, and demonstrated that the sites are saturable with an affinity constant of $8.7X10^{-7}$ M. However, isolation and characterization of a receptor-like protein from the target cell membrane has yet to be achieved. It appears that a better understanding of steroid binding specificities and the process of steroid binding

itself should help address this issue.

According to the free hormone hypothesis (Mendel, 1989), the biological activity of a hormone is modulated by the unbound (free), rather than protein-bound, concentration of hormone in plasma. This hypothesis is mainly based on a mathematical model derived from the different rate constants of the overall process. Particularly, in case of cortisol, there are very few supporting data. When free or CBG-bound cortisol was injected intravenously in the liver of a rat which has been adrenalectomized, no difference in cortisol effect was found as reported by Rosner and Hochberg (1972) and the authors concluded that CBG does not influence cortisol action *in vivo*. In a different study it was found that CBG-bound cortisol was biologically inactive (Slaunwhite et al., 1962).

Recent studies indicate that steroid hormones may have some rapid effects which can not be explained by the genomic mode of action (Majewska et al.,1986; Nabekura et al.,1986; Dluzen & Ramirez,1989; Hua and Chen, 1989). It was recently reported that progesterone can activate the calcium channel in the plasma membrane of human sperm within seconds (Blackmore et al., 1990). This emerging concept of non-genomic mode of action of steroid hormones further emphasizes a possible role of steroid binding proteins in regulating the action of steroid hormones. In this context, the report that steroid-bound CBG can actually activate a second messenger system by binding to specific membrane acceptor sites should be considered carefully (Rosner, 1990).

Role of glycosylation in biological activity of hCBG

The cDNA sequence of hCBG predicts a signal peptide of 22 amino acids. This implies that, during translation, the nascent protein is targeted to the membrane of the rough endoplasmic reticulum (RER), where the signal cleavage and core glycosylation occur. hCBG derived from human serum has complex-type oligosaccharides, indicating that further processing of carbohydrate occurs during transport through Golgi apparatus. However, the role of glycosylation in the biological activity of hCBG is less clear. Michelson et al. (1982) enzymatically deglycosylated hCBG and found that the full cortisol binding activity was retained as measured by equilibrium dialysis. There is some indication in recent studies, however, that variations in carbohydrate composition may modify the interactions between hCBG and target cell membranes during pregnancy (Avvakumov and Strel'chynok, 1988). In general, glycosylation exerts diversified effects on the structure and function of proteins (reviewed in Olden et al., 1982). So, it is of interest to examine 1) whether cotranslational N-glycosylation is required for the proper folding of hCBG to its active conformation and, 2) where in the secretory pathway nascent hCBG attains its fully active conformation.

Steroid binding site of hCBG

To understand the fundamental mechanism of protein recognition of glucocorticoids, hCBG appears to be the model system of choice compared to the intracellular glucocrticoid receptor, since the latter is a multidomain protein. The cDNA sequence of hCBG shows two cysteine residues in the mature protein (Hammond et al., 1987), and two sulfhydryl groups are actually titratable with 5.5'-

dithiobis(2-nitrobenzoic acid), after reduction and denaturation (Le Gaillard and Dautrevaux, 1978). Without reduction and denaturation, neither of these S-H group was detectable in cortisol-bound hCBG, by this method. When cortisol-bound native hCBG was reduced with dithiothreitol, however, one S-H group became titratable (Defaye et al., 1980), When cortisol was removed from cortisol-bound hCBG, furthermore, a second S-H group was detected (Defaye et al., 1980). It therefore appear that, out of the two cysteine residues in mature hCBG, one is in an oxidized form and is titratable upon reduction. The other sulfhydryl group is completely inaccessible to sulfhydryl reagents in cortisol-bound hCBG, and therefore, is likely to be in the cortisol binding site.

Defaye et al. (1980) further used spin labeled (C-17) steroid analogs with increasing distance of spin label from the steroid D ring, and investigated the topography of the steroid binding site by electron spin resonance measurements. This study revealed that (1) the steroid binding crevice has a total depth of 25 Å and (2) the C-17 side chain of steroid is directed towards the outside of the crevice with approximately 16 Å separating the C-17 from the opening of the crevice. Also, one thiol group was found at a depth of about 15 Å in cortisol free hCBG by using spin-labeled N-ethylmaleimide reagents of various side-chain lengths.

That a cysteine residue is directly involved in the steroid binding process was suggested by affinity labelling study with 6-β bromoprogesterone (Khan and Rosner, 1977). Affinity labeling involves two processes, binding followed by covalent bond formation. They demonstrated the following: (1) Binding of the steroid analogue to hCBG could be competed by ^{3}H-cortisol at early times but not

after 10 minutes, at which time a covalent bond has been formed between the analogue and S-H group; (2) one of the thiol groups is lost upon binding of the steroid analogue, as measured by the Ellman procedure; and (3) it is indeed a cysteine residue that is affinity labeled, as shown by thin layer chromatography of hydrolysates derived from the affinity complex.

Spectroscopic studies indicate that a tryptophan residue may also be involved in the steroid binding process (Akhrem et al., 1978). The excitation and emission properties of CBG are characteristic of a tryptophan-containing polypeptide. In addition, the fluorescence intensities of human and guinea pig CBG are quenched upon binding cortisol (Mickelson and Westphal, 1986). The quenching was proposed to be due to radiationless energy transfer from an excited tryptophan to the near-ultraviolet absorption band of steroid containing 3-oxo-4-ene group, as proposed for progesterone binding globulin (Stroupe et al., 1975). In hCBG, presence of two tryptophan residues (Trp 185 and Trp 266) within an evolutionary conserved domain further supports this hypothesis.

The present study describes the first reported expression of biologically active hCBG in a eucaryotic system, and provides evidence that cotranslational glycosylation is essential for the attainment of a biologically active conformation. Expression and characterization of mutant hCBGs also revealed that cys_{228} in hCBG is not directly involved in the steroid binding process. Furthermore, by chemical modification of the single cysteine-containing mutants, data have been obtained that implicate cys_{60} as being present near the steroid binding site and possibly involved in the binding process. Further analysis by site-directed mutagenesis and deletion

mapping of hCBG should enhance our understanding of the steroid binding site of this macromolecule.

III. MATERIALS AND METHODS

A. Isolation of hCBG cDNA From Human Liver cDNA Library in λ gt11

1. Preparation of λ gt11 plaques : Human CBG cDNA was isolated from a human liver cDNA library (Clonetech Laboratories Inc.) inserted in λ gt11 at the EcoRI site. To isolate viral plaques, cDNA was diluted serially in ice cold 10 mM Tris-HCl buffer, pH 7.5 containing 10 mM magnesium chloride and 100 uM EDTA. 1.2 ml of λ gtll dilution was mixed with 1.2 ml of *E. coli* (strain Y1090) grown overnight in LB medium containing 0.4% maltose at 37°C. The mixture was incubated at 37°C for 20 minutes and 200 µl was added to each LB-agar plate. Immediately, 3 ml agarose (0.7%, liquid at 55°C) was added on top and the plates were rocked gently to spread agarose. The plates were kept at room temperature to solidify the agarose and then were incubated at 37°C until plaques were visible (about 6 hours). About 150,000 plaques were spread on each plate.

2. Transfer of plaques to membrane : Nitrocellulose paper (BA85, Schleicher & Schuell) was placed on the plates for 30 seconds (first filter) and one minute (second filter). Immediately after transfer, all the filters were washed sequentially in the following solutions: 1) 1.5M sodium chloride, 0.5M sodium hydroxide for 30 seconds; 2) 1.5 M sodium chloride, 1M Tris-HCl, pH 8.0 for 2 minutes; 3) 2xSSC for 2 minutes. Filters were air dried and were baked in a vacuum oven at 80°C for two hours and kept at -20°C until use.

3. End labelling of oligonucleotides : Two oligonucleotides which were used as probes have the following sequences:

1. 5' TAT ACT GGA CAA TGC CAC TCC TCC T 3'

2. 5' CAG AGT TGT GTC TAA CTT TAG GCA T 3'

Oligo 1 represents bp 25-49, and oligo 2, bp 1371-1395, of the published sequence of hCBG cDNA (Hammomd et al., 1987). Oligonucleotide solutions were lyophilized several times and were finally dissolved in water. Oligonucleotides were 5' end-labeled with γ ^{32}P ATP according to the following method described by NEN/Du Pont. The reaction mixture contained 50 mM Tris-HCl pH 8.0, 10 mM magnesium chloride, 1.5 mM spermidine, 80 ng of oligonucleotide, 50 μCi γ ^{32}P-ATP (specific activity = 5000 Ci/mmol) and 3 units of T4 polynucleotide kinase (BRL) in a total volume of 50 μl and the reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 400 μl of reagent A supplied with NENSORB 20 nucleic acid purification cartridge (NEN/Du Pont) which contains 100 mM Tris-HCl pH 7.7, 10 mM triethylamine and 1 mM EDTA. The labeled products were purified by passing through the cartridge following the manufacturer's protocol. The specific activities of the labeled 5' and 3' oligonucleotides were $3x10^8$ and $7X10^7$ cpm per μg DNA, respectively.

4. Hybridization : Hybridization of the ^{32}P-labeled synthetic oligonucleotides to the immobilized viral plaques on nitrocellulose membrane was carried out according to the method described by Maniatis et al., (1982). Nitrocellulose filters were washed in 3xSSC (20xSSC= 3M sodium chloride, 0.3M sodium citrate)/0.1% Sodium dodecyl sulfate (SDS) at 60°C for 30 minutes and were then transferred to prehybridization solution which contained 6xSSC, 5x Denhardt's solution (50x solution= 10 μg/ml Ficoll, 10 μg/ml polyvinylpyrrolidone and 10 μg/ml

BSA), 0.5% SDS, 0.05% sodium pyrophosphate and 100 μg/ml salmon sperm DNA. Prehybridization was carried out in a sealed bag at 37°C for 90 minutes. For hybridization, filters were then taken in another sealed bag containing 25 ml of solution containing 6xSSC, 1x Denhardt's solution, 100 μg/ml of yeast tRNA, 0.05% sodium pyrophosphate. ^{32}P-labeled probes were boiled for 2 minutes and added to the the solution at a concentration of 10^5 cpm per ml. Hybridization was carried out overnight at 50°C. Filters were then washed in 6xSSC/0.05% sodium pyrophosphate at room temperature for 15 minutes and were then exposed to X-ray film at -70°C.

A single plaque, which hybridized to both probes, was further purified by secondary and tertiary plaque screening. Each of the final plaques was suspended in 1 ml dilution buffer and one drop of chloroform, kept at room temperature for an hour, and was then stored at 4°C.

5. Preparation of stock of λ phage and phage DNA : A small scale stock of λ phage was made by growth in *E. coli* Y1090 (grown overnight in o.4% maltose containing LB medium) in plates with top agarose (0.7%) and scraping the plaques with dilution buffer. A few drops of chloroform were then added to the suspension and incubated 10 minutes. The supernatant was collected after centrifugation at 10,000 rpm in JA-20 Beckman rotor for 10 minutes. For making a liquid lysate , 500 μl ($5X10^7$ plaque forming units) of small scale stock was mixed with 2.4 ml *E. coli* Y1090 culture (10^{10} bacteria) and incubated at 37°C for 20 minutes, shaking frequently. The mixture was then added to 500 ml NZC medium (NZC medium= 10g NZ amine A, 5g sodium chloride, 2g magnesium chloride and 1g casamino acid per liter) and incubated with vigorous shaking at 37°C until

bacterial lysis occured. A few drops of chloroform were added to lyse any remaining cells and the solution was incubated 30 minutes at 37°C. To 500 ml liquid lysate, pancreatic DNase I and RNase were added each at final concentration of 1 μg/ml. After 30 minutes incubation at room temperature, 29.2 gms of sodium chloride was added, dissolved by swirling and incubated on ice for an hour. The bacterial debris was removed by centrifugation. To the clear supernatant, 50 gms polyethylene glycol (PEG 8000) was added and dissolved by swirling. The suspension was kept at 4°C overnight. The phage particles were pelleted by centrifugation at 11,000g for 10 minutes at 4°C. Resuspended phage particles in dilution buffer were extracted with a equal volume of phenol and chloroform and layered on a CsCl step gradient (with a density range of 1.3 to 1.7 g/ml) in SW27 tubes. Centrifugation was at 24,000 rpm (SW27 rotor) at 4°C for two hours. The isolated phage containing band was diluted in 1.5 g/ml CsCl in dilution buffer in a Ti50 tube, and the phage band was visible after centrifugation at 38,000 rpm for 24 hours. The phage band was isolated and the phage containing solution was dialyzed for one hour, extracted with phenol/chloroform and dialyzed against TE buffer to remove the residual phenol/chloroform. The DNA was further purified by ethanol precipitation. The viral DNA was digested with EcoRI and 1.4 kb cDNA of hCBG was directly isolated from the low melt agarose gel.

B. Cloning, Transformation and Large Scale Plasmid DNA Preparation

1. Dephosphorylation of vector DNA: hCBG cDNA was cloned in pGEM1 vector (Promega) by standard techniques (Maniatis et al., 1982). The vector

DNA was linearized with EcoRI and the linear vector DNA was dephosphorylated in a 100 µl reaction of 18 µg of linear DNA, 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA and 0.5 unit of calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals). The reaction was carried out at 37°C for 30 minutes and was stopped by adding 20 µl of 250 mM EGTA and then heating at 68°C for 45 minutes. The mixture was then extracted with phenol/chloroform. The DNA was finally purified by ethanol precipitation.

2. Ligation and transformation : hCBG cDNA in low melt agarose was ligated to the above vector DNA in a ligation reaction containing 66 mM Tris-HCl, pH 7.5, 5 mM magnesium chloride, 1mM dithiothreitol (DTT), 1 mM ATP, 200 ng of vector DNA, 200 ng of cDNA and 1 unit of T4 DNA ligase (Boehringer Mannheim Biochemicals). The reaction was carried out at 25°C for one hour. *E. coli* (strain MC1000) was transformed with the ligation mixture and the transformed colonies of bacteria were screened by their growth in ampicillin containing agar (40 µg/ml). With the small scale DNA preparation of the transformed colonies, a restriction analysis was performed to identify a colony transformed with the recombinant plasmid vector containing hCBG cDNA oriented 5' to 3' with respect to the T7 promoter.

3. Extraction and purification of plasmid DNA : A large scale preparation of pGEM1-CBG plasmid DNA was made by the alkaline lysis method and purified from a CsCl gradient as described by Maniatis et al (1982).

4. Sequencing : The 5' end of the cDNA was sequenced by dideoxynucleotide chain termination method (Sanger et al., 1977) using modified T7

DNA polymerase (Sequenase, United States Biochemicals system) and T7 promoter sequence as primer (Promega).

C. *In Vitro* Transcription and Translation

1. Transcription : The recombinant pGEM1-CBG was linearized with Pst1 (present in the vector sequence), and the cDNA in the linear plasmid was transcribed under the control of T7 promoter as follows: A typical 50 µl transcription reaction contained 5µg of linear DNA, 40 mM Tris-HCl, pH 7.5, 6 mM magnesium chloride, 2 mM spermidine, 10 mM sodium chloride, 10 mM DTT, 20-40u of RNase inhibitor, 500 µM rNTPs and 80U of T7 RNA polymerase (Bethesda Research Laboratories). The reaction proceeded for one hour at 37°C, after which 10µl (10units) of RQ1 DNase were added. The reaction was carried out for an additional hour at 37°C. The resulting mRNA was extracted with phenol/chloroform and purified by ethanol precipitation.

2. Translation : The *in vitro* transcribed hCBG mRNA was translated in the rabbit reticulocyte lysate system (Promega). This lysate was pretreated with micrococcal nuclease to destroy endogenous mRNA and was supplemented with an energy generating system (phosphocreatine kinase & phosphocreatine), a mixture of calf liver tRNA, hemin to prevent inhibition of initiation, and potassium and magnesium salts in optimum concentration for translation of most mRNA species. A standard 50 µl reaction mixture contained 35 µl lysate, 1 mM amino acid mixture lacking methionine, 50 µci ^{35}S-methionine (specific activity 1000 Ci/mmol, ICN Biochemicals), and 2 µg mRNA stock. The reaction was carried out for one hour

at 30°C. Incorporation of ^{35}S-methionine into protein was monitored by precipitation in 10% TCA and hot 5% TCA. Canine microsomal membranes, prepared by the method of Shields and Blobel (1978), were included in the standard translation reaction in order to follow the processing of the precursor nascent polypeptide, such as signal cleavage and core glycosylation.

D. Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis

1.Immunoprecipitation : Immunoprecipitation was carried out as described (Caplan et al., 1991). *In vitro* translated products or cell lysates were brought to 2.5% SDS and boiled for 3 minutes, and then they were diluted with antibody buffer A (190 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 6 mM EDTA. 2.5% Triton X-100, 2 mM PMSF, 1 mg/ml BSA, 10 mM methionine). 5-10 µl of rabbit anti-hCBG antibody was then added to each tube. The reaction was then incubated overnight with gentle agitation at 4°C. Immune complexes were harvested by incubation with protein A Sepharose-CL 4B (Sigma), for 90 minutes at 4°C. Medium samples were processed and immunoprecipitated the same way, except that the samples were not boiled with SDS. The hCBG antibody from rabbit used throughout this work was a generous gift from Dr. Robert W. Kuhn (OBS/GYN, UCSF, California). The Sepharose beads were collected by centrifugation and were washed three times with buffer B (150 mM sodium chloride, 0.1% Triton X-100 V/V, 10 mM Tris-HCl, pH 8.3, 5mM EDTA, 2 mM PMSF, supplemented with 1mg/ml BSA and 10 mM methionine), and one time with phosphate buffered saline (PBS).

2. SDS-polyacrylamide gel electrophoresis : Direct samples or immune

complexes bound to protein A sepharose beads were boiled with SDS-PAGE loading buffer (1X buffer= 5% SDS, 50 mM DTT, 50 mM Tris base, 0.01% Bromophenol blue) for 3 minutes and were alkylated with 100 mM iodoacetamide for 20 minutes at 37°C in the dark. After centrifugation to remove the beads, the supernatants were applied to a 12.5% SDS-polyacrylamide gel. The gels were fluorographed with 22% (W/V) 2,5 diphenyl oxazole (Sigma) in dimethyl sulphoxide (Sigma), dried under vaccuum and exposed to Kodak XAR film at -70°C. The exposed films were developed in a XOMAT.

E. Treatment With Endoglycosidase H and N-Glycanase

1 Endoglycosidase H : Immune complexes on protein A Sepharose beads were treated with Endo H as follows: a standard 25 µl reaction mixture contained 0.5% SDS (W/V), 100 mM sodium phosphate, pH 5.5, 4 mM 1,10 phenanthroline monohydrate, and 4 mU of Endoglycosidase H (Genzyme) and the reaction was incubated for 2 hours at 37°C.

2. N-glycanase : A 30 µl reaction mixture contained 400 mM sodium phosphate, pH 8.6, 20 mM 1,10 phenanthroline, 0.18% (V/V) Nonidet P 40 (NP-40) and 0.3 units of N-Glycanase (Genzyme). The reaction proceeded for 18 hours at 37°C.

F. Cloning of hCBG cDNA in Baculovirus Derived Vector pVL1393

Cloning of the cDNA in baculovirus derived vector pVL1393 (Invitrogen) at the EcoRl site (present at the downstream of the polyhedrin

promoter) was performed as described above for pGEM1. For transformation of ligation products, *E. coli* MM294 strain was used. A large scale plasmid DNA was made from the resulting pVL1393-CBG.

G. Production of Recombinant Baculovirus

1. Transfection : *Sf9* cells were transfected with wild type AcMNPV viral DNA and recombinant pVL1393-CBG plasmid DNA as described (Summers and Smith, 1987). $3x10^6$ cells in a 25 cm^2 flask were covered with 0.75 ml of Grace's medium containing 10% FBS and 50 ug/ml of gentamycin. 1 µg of AcMNPV DNA and 3.15 µg of pVL1393-CBG DNA were mixed in 0.75 ml transfection buffer (25 mM Hepes, pH 7.1, 140 mM sodium chloride, 125 mM calcium chloride) and added dropwise to the cells in the flask. The cells were incubated for 4 hours at 27°C. The viral inoculum was then replaced with TNMFH (10% FBS). After four days of incubation, the medium was collected, cells were pelleted and the supernatant was stored at 4°C. To determine the viral titer, *sf9* cells were infected with serially diluted virus in a 96 well plate and incubated for 5 days. The dilution at which 50% of the wells contained infected cells was used for calculating the number of plaque forming unit (pfu) per ml of stock transfection medium.

2. Screening of recombinant virus : Primary screening for recombinant virus was done by cDNA probe hybridization of viral plaques (Summer and Smith, 1987). Briefly, *sf9* cells were infected with 3 different dilutions of transfection medium (1:10, 1:100, 1:1000). Since the viral titer was $2x10^8$, at the highest dilution, I screened 800,000 plaques, which I expected to be sufficient to obtain a good

number of recombinant virus. Seven days after infection, a replica plate was made from each plate and the remaining cells and medium were treated with 0.5M sodium hydroxide for 20 minutes and then transferred to Zeta probe membranes (Bio-Rad). Membranes were washed with 2xSSC (1xSSC= 0.15M sodium hydroxide, 0.015M sodium citrate) and were air dried. hCBG cDNA was labeled with α^{32}P-dCTP (3000 Ci/mmol) by random primer labeling (New England Nuclear) according to the manufacturer's instructions, to a specific activity of $1.5x10^8$ cpm/μg DNA.

Hybridization was done according to the method of Maniatis et al (1982). Positive plaques were further amplified and subjected to a second round of screening. This was done by the plaque purification method described by Summers and Smith (1987). This method is based on the distinct morphology of recombinant virus-derived plaques. In *sf9* cells infected with wild type virus, expression of the polyhedrin protein results in occlusion bodies which are clearly visible under a dissecting microscope. In the recombinant virus, the polyhedrin gene is interrupted by insertion of a foreign gene, and plaques derived from these viruses lack occlusion bodies and look less refractile. Plaques were formed by infecting *sf9* cells with partially purified recombinant virus in 60 mm plates and the cells were covered with TNMFH (10% FBS) medium containing 1.5% low melt agarose (Sea Plaque, FMC Bioproducts) after the infection period. Uninfected and wild type virus infected cells were processed in the same way to serve as controls. Of the many apparent recombinant plaques picked, only a few were further plaque-purified to obtain a plate containing only occlusion-negative plaques. From a single occlusion-negative plaque, a large stock of reombinant virus (AcMNPV-CBG) was made.

H. Cell Culture and Medium

Spodoptera frugiperda (*sf9*) cells were maintained in Grace's insect cell culture medium (Gibco) supplemented with TC yeastolate, lactalbumin hydrolysate (each at 3.33 gms/L, Difco) and 10% fetal bovine serum (Gibco). This medium is known as TNMFH (10% FBS). In order to grow cells in serum-free medium, 1 ml of ITS supplement was added per 100 ml Grace's medium containing TC yeastolate and lactalbumin hydrolysate or directly sf900 (Gibco) medium was used. ITS suppliment contains insulin, transferrin, selenious acid, and BSA (Collaborative Research product). *Sf9* cells were always maintained at 27°C.

I. Infection and Metabolic Labelling of *Sf9* Cells

$1.5X10^5$ *sf9* cells in 35 mm plates were infected with 1 ml viral dilution in TNMFH medium (10% FBS) at a multiplicity of infection of 10-25 per cell for 90 minutes at 27°C. The medium was then replaced with 3 ml TNMFH (10% FBS). After 2-3 days of infection, the old medium was discarded and 1 ml of labeling medium was added. Labeling medium lacks unlabeled methionine and contains 100 μCi of ^{35}S-methionine per ml. At intervals, the medium was then collected and cells were lysed in 100 μl of PBS (25 mM phosphate buffer pH 7, 125 mM sodium chloride) containing 0.5% (V/V) NP-40 and 0.5% (W/V) sodium deoxycholate. The post-nuclear supernatant was collected.

J. Immuno Quantification of Expressed hCBG in *Sf9* Cells

The assay is based on the observation that the anti-hCBG antibody

recognizes hCBG derived from any of the following; *in vitro* translation product, recombinant virus infected *sf9* cells and human serum. The ability of the *sf9*-derived and human serum-derived hCBG to compete in immunoreaction with a fixed amount of radiolabeled *in vitro* translated product was compared. The amount of hCBG present in *sf9* cell lysate was calculated from the standard curve produced with pure hCBG.

The immunoreaction was carried out with limiting anti-hCBG antibody. $2x10^5$ cpm of SDS-denatured translation products were diluted with 1 ml antibody buffer A followed by addition of 5 µl of antibody (50x diluted immune serum of rabbit raised against pure hCBG). Increasing amounts of either pure hCBG (0-500 ng), human pregnancy serum (0-20 µl) or cell lysate (0-10 µl) were then added. Immunoprecipitation was carried out as described above and the immunoprecipitates were resolved on 12.5% SDS-PAGE. Linear exposures of the autoradiograms of the gels were scanned in a densitometer (Ultroscan XL, Enhanced laser densitometer, Pharmacia LKB). The values were plotted against the amount of samples used in each assay.

K. Immunoblot Analysis

Immunoblotting was performed as described below (Harlow & Lane, 1988). Proteins derived from cell lysate or medium were resolved in SDS-PAGE, and were electrophoretically transferred to nitrocellulose membrane using the Bio-Rad semi-dry gel transfer apparatus. Membranes were blocked with 5% nonfat dry milk in TBST (100 mM Tris-HCl, pH 7.4, 150 mM sodium chloride and 0.05% Tween 20)

and then incubated overnight with rabbit anti-hCBG antibody (diluted 1:500) at 4°C. After several washes with TBST, membranes were incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (diluted 1:1000) in TBST for one hour. For color development, membranes were then transferred to a solution containing 66 µl nitro blue tetrazolium, 33 µl 5-Br-4Cl-3 iodonyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM sodium chloride, 5 mM magnesium chloride. The reaction was stopped by adding 20 mM Tris-HCl, pH 8.0, 5 mM EDTA.

L. Steroid Binding Assay and Scatchard Analysis

1. Steroid binding : This assay involves two steps: (1) Binding of ^{3}H-cortisol to hCBG and (2) binding of the ^{3}H-cortisol-hCBG complex to DEAE ion exchange filters (Schiller and Petra, 1976; Ross et al., 1982). The cortisol binding capacity of human pregnancy serum, cell lysates, or medium was assayed as follows: the samples were diluted with 10 mM Tris-HCl, pH 7.4. 150 µl of each sample was then incubated with 5 pmol of ^{3}H-cortisol (with/without 500 pmol of unlabeled cortisol) for 2 hours at 4°C. 50 µl of the incubation mixture was then applied to DEAE ion exchange filters (DE 81, Whatman), the filters were washed by vacuum filtration eight times with 1 ml aliquots of buffer, and the filters were counted directly in Econofluor (NEN) in a scintillation counter. Cortisol binding to hCBG has a pH optimum between 8 and 11 (Westphall, 1986). At pH 7.4, the dissociation constant is in the range 10^{-8} to 10^{-9}M. ^{3}H-cortisol remains associated tightly with filter bound hCBG during the washing procedure, whereas there is a high rate of dissociation from the low affinity binding sites. Non-specific binding was obtained

from the bound cpm in the presence of 500 pmol of unlabeled cortisol. The specifically bound ^{3}H-cortisol was calculated by subtracting the non-specific binding from the total binding.

2. Scatchard analysis : To determine the K_d of hCBG, a Scatchard analysis (Scatchard, 1949) was performed. Samples were incubated with increasing amounts of ^{3}H-cortisol(0.5-10 pmol) in the absence or presence of 100-fold excess unlabeled cortisol. The incubation and processing of the filters were done as stated above. The amount of free ^{3}H-cortisol was calculated by subtracting the specifically bound cpm from the total cpm added to each assay. According to the relationship,

$$\frac{B_{obs}}{F} = \frac{B_{max}}{K_d} - \frac{B_{obs}}{K_d},$$

where B and F are bound and free ligand, respectively, bound/free cortisol was plotted against the bound cortisol concentrations. The K_d was obtained from the best straight-line fit to the data, as determined by linear least square analysis.

M. Site-Directed Mutagenesis

1. Design and synthesis of oligonucleotides : Oligonucleotide-directed mutagenesis was carried out in a two step polymerase chain reaction as described by Landt et al.(1990) and as outlined in Fig. 9. Mutagenic oligonucleotides were designed to change cys_{228} to serine or alanine. The following sequences represent the two synthetic oligonucleotides, which correspond to bp 771 to 797 of the cDNA in

the antisense direction:

Oligo 3: 5' CTG CAC CAG CTG GCT GGG GAG CTC TGA 3'

Oligo 4: 5' CTG CAC CAG CTG GGC GGG GAG CTC TGA 3'

Oligo 3 and 4 contain the codon for Serine and alanine in place of cys_{228} codon, respectively. The change in base(s) within these codons are underlined. The melting temperatures of these oligonucleotides, calculated according to the formula $T_m = 2 (A+T) + 4 (G+C)$, are 90°C for oligo 3 and 92°C for oligo 4.

The sequence of the universal 5' primer was 5' GCC AGC AGA CAG ATC AAC 3' (Oligo A) and this sequence represents bp 531 to 548 of the sense strand of the cDNA. The 3' end of this primer is 222 bp away of the 3' end of oligo 3 and oligo 4. Sequence of the universal 3' primer was 5' TCG TGT CCC GGC TCA GTG 3' (Oligo B) and this sequence represents the bp 871 to 888 of the cDNA in the antisense direction. The 3' end of this primer is 73 bp away from the 5' end of oligo 3 and 4. The 5' and 3' universal primers flank the Sal 1 and BspE 1 restriction sites, respectively, so the final PCR fragments could be recloned at those sites. The overall strategy has been outlined in Fig. 9.

2. Polymerase chain reaction (PCR) : PCR was carried out in a DNA thermal cycler (Perkin Elmer Cetus). The first round of PCR was performed in 100 µl 1X Taq polymerase buffer containing 2 µM oligo A, 2 µM mutagenic oligonucleotide (oligo 3 or 4), 2 ng of pGEM1-CBG DNA, 50 µM dNTP mixture, and 10 units of Taq DNA polymerase (Promega). The reaction mixture was overlayed with mineral oil. Each of the 30 cycles consisted of 60s at 94°C (denaturation), 60s at 55°C for oligo 3, at 58°C for oligo 4 (annealing), and 45s at

72°C (primer extension). The resulting intermediate fragments were gel purified (Maniatis et al. 1982). The second PCR reaction was carried out as described for the first PCR but at this time the entire intermediate fragment served as the 5' primer and oligo B was used as the 3' primer. Also the annealing temperature was changed to 45°C.

3. Ligation and cloning of PCR fragments : Products from the second PCR were digested with Sal 1 and BspE 1, and the fragments were gel purified. These fragments containing the desired mutation were then reconstituted into pGEM1-CBG in two steps as follows: EcoRI/Sal 1 and BspE I/EcoRI fragments of the wild type cDNA were gel purified and these two pieces of DNA fragments were ligated first to the restriction digested, gel purified PCR fragment. The ligation protocol was as before, and the incubation was carried out for 1 h. EcoRI linearized pGEM1 vector DNA was then ligated to the reconstituted cDNA containing the desired mutation, in a second step. The presence of desired mutations were confirmed by DNA sequencing of the region overlapping the Sal 1 and BspE 1 sites by using pGEM1-CBGS228 and pGEM1-CBGA228 as target DNA and oligo A as the primer. All the sequencing was done in an Applied Biosystem 373A DNA sequencer in the DNA core laboratory of the Brookdale Center for Molecular Biology (Mount Sinai School of Medicine). The above mentioned oligonucleotides were also synthesized by the DNA core center using the Applied Biosystems 380B DNA synthesizer.

1.4 Kb Eco RI inserts containing the mutated cDNAs were then cloned in the baculovirus derived vector pVL1393, to make the recombinant vector

pVL1393-CBGS228 and pVL1393-CBGA228. Recombinant baculovirus (containing the mutated cDNA sequence) stocks were made by transfection as before and the resulting recombinant viruses were designated as AcMNPV-CBGS228 and AcMNPV-CBGA228 (containing the serine and alanine mutations, respectively).

N. Chemical Modification of hCBG with Sulfhydryl Specific Reagents

Sf9 cell derived medium containing wild type or mutant hCBGs was concentrated several fold, and brought up in 10 mM Tris-HCl, pH 8.0, to achieve a pH of 8.0. The final samples contained about nM hCBG. 320 µl of each sample were then treated with 50 mM DTT for 1 h at room temperature. The reduced samples were then treated with 200 mM N-ethylmaleimide (Roberts and Rouser, 1958), 100 mM iodoacetamide (Lundblad and Noyes, 1988) or 200 mM sodium tetrathionate (Liu, 1967) for different times at room temperature, in the dark. Excess reagents in the samples were removed by passing through a spin column composed of Sephadex G-25. The eluate was directly taken for assay of ^{3}H-cortisol binding.

CHAPTER I

In Vitro and *In Vivo* Expression of hCBG

(Results of Chapter I has been published: Ghose-Dastidar, J., Ross, J.B.A., and Green, R. (1991) Proc. Natl. Acad. Sci. USA 88:6408-6412)

Introduction

To understand the biogenesis of hCBG, I have expressed hCBG *in vitro* in the rabbit reticulocyte lysate system and *in vivo* in the baculovirus/insect cell system. The goal was to understand how this macromolecule achieves its final steroid binding conformation by comparing the steroid binding properties of hCBG derived from these two systems. The long term goal of the expression of hCBG in insect cells is to produce large amount of wild type and mutant proteins for physico-chemical analysis of the steroid binding site. hCBG contains six consensus sites for N-linked glycosylation, of which five are actually modified in the native protein. However, the role of glycosylation in the biogenesis or function of hCBG was not known. Since these sites are conserved in CBG from different species, the question arises whether modification by N-linked glycosylation has any effect on the structure or function of hCBG. Successful expression of hCBG in *sf9* cells allowed me to address this issue through production of unglycosylated hCBG.

Results and Discussions

A. Isolation of hCBG cDNA From Human Liver cDNA Library /Identification by Restriction Mapping and Partial sequencing

My first goal was to isolate a full length cDNA encoding hCBG. For this purpose, a human liver cDNA library constructed in λ gt11 was screened. Based on the published sequence of hCBG, two oligonucleotides were synthesized, sequences of which were derived from the 5' and 3' region of the cDNA. Both of the 5' and 3' end probes were used in first two rounds of screening after 5' end-labelling

with ^{32}P. In the primary screening 150,000 plaques were analyzed, and a single plaque was hybridized with both probes (Fig. 1, a and b). The hybridized plaque was further subjected to plaque purification. Successful purification was confirmed by a tertiary screen, in which all plaques were hybridized with radiolabeled probe.

A single plaque was isolated from this plate and checked for the presence of hCBG cDNA by EcoRI digestion of viral DNA. Restriction digestion showed the expected 1.4 Kb band (Fig. 2b). The cDNA was then cloned into the pGEM1 vector at the EcoRI site in 5' to 3' orientation with respect to the T7 promoter. Further restriction digestion was performed to check the identity of the cDNA (Fig. 2b), the restriction map was as predicted from the cDNA sequence. Partial sequencing of the 5' end of the cDNA was performed by the chain termination method using dideoxy nucleotides (Sanger et al., 1977) and the T7 promoter primer. This demonstrated that the cDNA starts 35 bp upstream of the translational initiator codon (data not shown).

B. *In Vitro* Synthesis of Immunologically Active hCBG: Characterization of Different Forms

hCBG cDNA was cloned in the PGEM1 vector to place it under the control of the T7 promoter. Recombinant vector DNA (pGEM1-CBG) was then linearized with Pst 1 and transcribed with T7 RNA polymerase. The *in vitro* transcribed hCBG mRNA was translated in the presence of ^{35}S-methionine in the wheat germ (WG) or in the rabbit reticulocyte lysate (RRL) cell free system, and the translation products were immunoprecipitated with anti-hCBG antibody and resolved

by 12.5% SDS-PAGE (Fig. 3). A major band at M_r 42 KD was observed (Fig. 3, lane 1) along with some minor lower M_r forms in both systems. When canine microsomal membranes (derived from rough endoplasmic reticulum) were included in the RRL translation reaction, a few higher M_r forms are observed (Fig. 3, lane 2). Notably, no such higher M_r forms could be detected in the WG-derived translation products (data not shown).

To test if the higher M_r forms of hCBG are located in the lumen of the microsomal vesicles, a protection assay was performed. Only the higher M_r forms are protected from trypsin digestion (Fig. 3, lane 4). However all the higher and lower M_r forms disappear when detergent was included in the digestion mixture (Fig. 3, lane 3). When these protected forms are treated with Endo H, two low M_r forms appear, along with the complete disappearance of the higher M_r forms (lane 5). The upper band in lane 5 is probably the signal cleaved, partially deglycosylated form.

From these results, several conclusions were reached. (1) The cDNA sequence that has been isolated is identified as hCBG cDNA, as observed by the recognition of the translated product by anti-hCBG antibody and by the molecular weight of the precursor polypeptide. (2) The primary translation product contains the amino terminal signal sequence of hCBG which targets the nascent polypeptide to the membrane of RER and allows translocation. (3) The higher M_r translation products observed in presence of microsomes are in fact located in the lumen of RER, as evidenced by the protection of these forms from trypsin digestion. Only treatment with trypsin in presence of detergent, which permealizes the membrane, can degrade these forms. (4) These membrane protected forms are further

characterized as high mannose containing glycosylated forms of hCBG by treatment with endoglycosidase H (Endo H) (which cleaves only high mannose-containing oligosaccarides). Endo H reduces the higher forms to two lower M_r forms. The lower one (38 KD) corresponds to the signal-cleaved form of hCBG.

Why glycosylation was not observed when translation was carried out in the WG system remains unexplained.

C. Expression of High levels of hCBG in the Baculovirus/Insect Cell System: Characterization of Recombinant hCBG

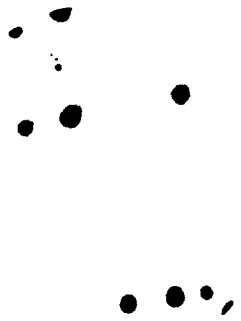
Rationale for choosing baculovirus/insect cell system : A wide variety of mammalian genes have been expressed in *Spodoptera frugiperda (sf9)* insect cells infected with recombinant *Autographa californica nuclear polyhedrosis* virus (AcMNPV) (Luckow & Summers, 1988). There are several reasons for using this system. First, it was evident from studies of several expressed proteins that co- or post-translational modifications such as signal cleavage, core glycosylation, phosphorylation, disulfide bond formation and specific proteolytic cleavage can take place in these insect cells. Second, high level synthesis and secretion of many foreign genes have been shown to occur under the control of the strong baculovirus polyhedrin promoter (Luckow and Summers, 1988). Third, in the recombinant virus, the viral protein polyhedrin coding sequence is interrupted by inserting a foreign gene in this region. Viral plaques derived from the recombinant virus therefore have a distinct morphology (due to the absence of occlusion bodies formed by polyhedrin). This allows the visual screening of the recombinant virus-derived plaques.

Figure 1: Isolation of hCBG cDNA from human liver cDNA library. Viral plaques prepared from a human liver cDNA library in λ gt11 were hybridized with ^{32}P-labeled oligonucleotide probes derived from 5' (a) and 3' (b) regions of the cDNA in the primary screen. The single plaque which hybridized to both probes was further plaque purified (secondary and tertiary screen).

PRIMARY SCREEN

a b

SECONDARY SCREEN



TERTIARY SCREEN

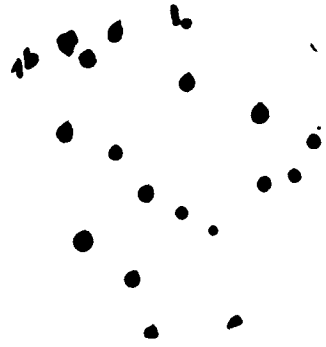
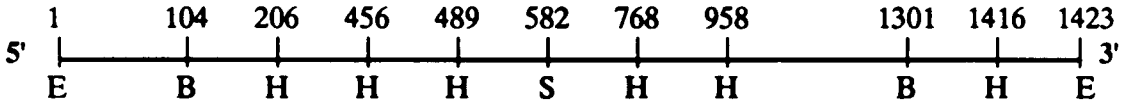
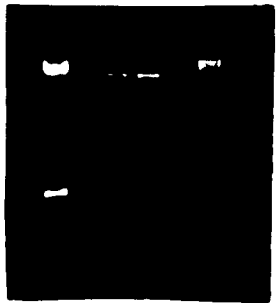


Figure 2. **Identification of hCBG cDNA .** Isolated hCBG cDNA was amplified by cloning in the pGEM1 vector at its EcoRI site. a) partial restriction map of hCBG cDNA. b) The resulting pGEM1-CBG was digested with the restriction enzymes as indicated (lanes 2-5).

a.



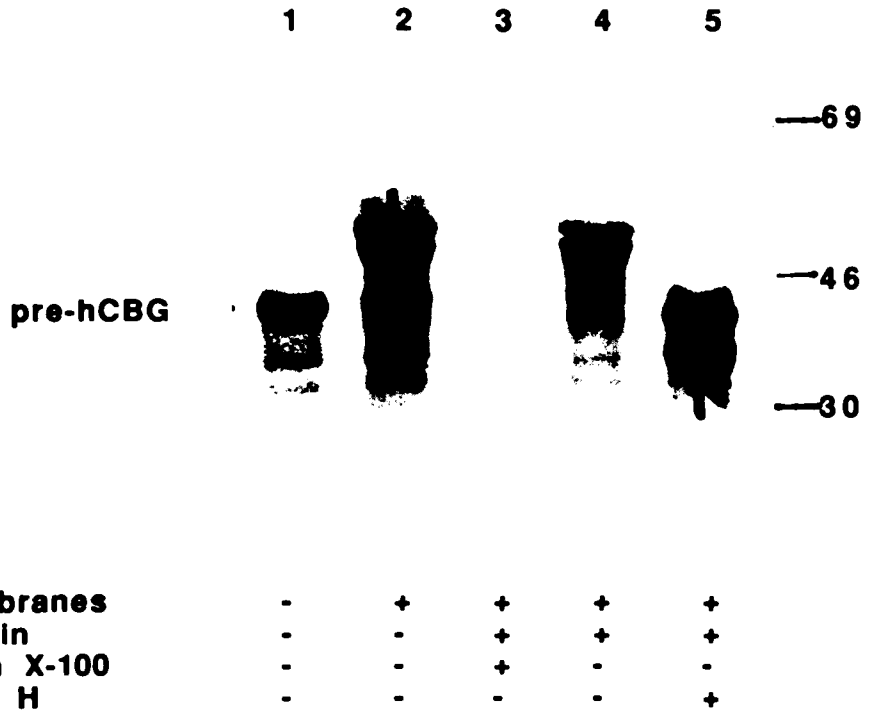
b



lanes

1. 123 bp DNA ladder
2. Eco RI
3. Eco RI + Bam HI
4. Eco RI + Sal I
5. Eco RI + Hinf I
6. I kb DNA ladder

Figure 3. ***In Vitro* Synthesis of Immunologically active hCBG: Characterization of Different Forms.** pGEM1-CBG was linearized with Pst 1 and transcribed with T7 RNA polymerase. The resulting mRNA was translated in rabbit reticulocyte lysate system in the presence of ^{35}S-methionine, in absence (lane 1) and presence (lanes 2-5) of canine pancreatic microsomes (membrane). After translation, the reaction products were incubated with trypsin (lanes 3-5). In lane 3, 0.5% (v/v) Triton X-100 was included. Immunoprecipitation was carried out by incubation with anti-hCBG antiserum. In lane 5, the immunoprecipitates were further digested with endoglycosidase H (Endo H). Immunoprecipitates were resolved on 12.5% SDS-PAGE.


1
2
3
4
5
69
46
30
pre-hCBG
membranes - + + + +
trypsin - - + + +
triton X-100 - - + - -
Endo H - - - - +

The baculovirus-derived vector pVL1393 was chosen because it allows production of nonfused protein. The authentic polyhedrin ATG codon has been changed to ATT, so that the first ATG of the foreign sequence will be the initiator codon. Furthermore, a multiple cloning site 35 base pairs downstream of that polyhedrin ATT is located where the foreign sequence can be inserted. The transcript of the foreign gene sequence will have the sequence immediately upstream sequence of the polyhedrin initiator ATG, which appears to be required for optimum expression of a foreign sequence (Luckow & Summers, 1989). The polycloning site contains a unique EcoRI site where hCBG cDNA could be directly inserted without further modification.

Specific expression of hCBG sequence in sf9 cells : To achieve the expression of hCBG under the control of baculovirus polyhedrin promoter, the 1.4 Kb cDNA of hCBG was cloned at the unique EcoRI site, and the recombinant virus AcMNPV-CBG was produced by transfecting *sf9* cells with the AcMNPV viral DNA and recombinant pVL1393-CBG DNA. A stock of recombinant virus was made by infecting *sf9* cells with AcMNPV-CBG.

In order to observe specific expression of hCBG, *sf9* cells were infected with the recombinant virus, and the cells were metabolically labeled with ^{35}S-methionine after 48-72 hours of infection (Fig. 4). The cell lysate and medium samples were immunoprecipitated with anti-hCBG antibody. Wild type virus-infected cells or uninfected cells were processed in the same way as controls.

Expression of hCBG occurs only in cells infected with the recombinant virus (Fig. 4a, the wild type virus control not shown). hCBG products are identified

by their binding to anti-hCBG antibody and their molecular weight. There is a band at approximately 42 KD which corresponds to the precursor form of hCBG (lane 4). In addition, there are several higher M_r forms (46-49 Kd) (lane 4). When these samples were treated with endoglycosidase H (Endo H), all of the higher M_r forms disappear, and at the same time a lower M_r form of 38 KD appears (lane 5), which migrates identically to the *in vitro* translated product which had been digested with Endo H (lane 3). Also, the higher M_r forms are sensitive to N-glycanase treatment, which produces a faster migrating band than that produced by Endo H digestion (lane 6). Only the 42 KD precursor form remains insensitive to those treatments, which is expected (lanes 5 and 6). Pretreatment of the cells with tunicamycin almost completely abolishes the higher M_r forms (Fig. 4b, lane 3) and there is a proportionate increase in the precursor and signal-cleaved form in the secreted material. Importantly, expressed hCBG is efficiently secreted into the medium (Fig. 4b). The secreted hCBG remains sensitive to Endo H (data not shown).

These results indicate that in *sf9* cells infected with the recombinant AcMNPV-CBG virus, there is specific expression of hCBG. The signal sequence present in hCBG is recognized by the insect cell translocation apparatus, as indicated by the efficiency of secretion. This sequence is appropriately cleaved by the insect cell signal peptidase as is evident from the appearance of a smaller M_r form upon digestion with Endo H. The sensitivity of the higher M_r forms to Endo H shows that these are high mannose oligosaccharide containing species. As reported in the literature (Luckow and Summers, 1988), many of the foreign glycoproteins expressed in these insect cells are smaller than their native counterparts, and also many of them

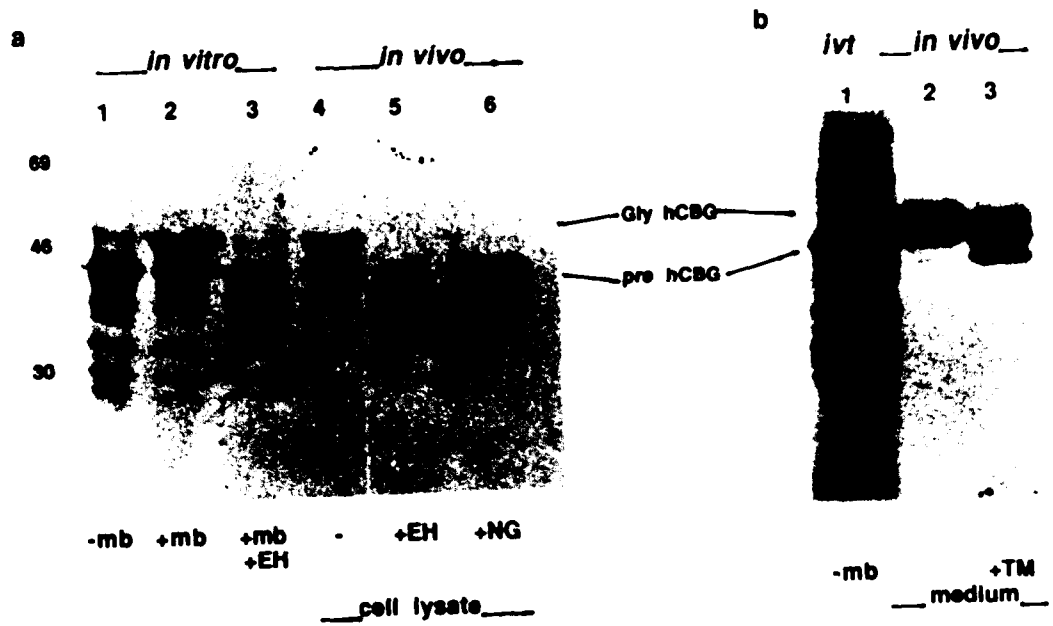
remain sensitive to Endo H. So, it is suspected that in these cells high mannose oligosaccharides are not generally further processed to complex oligosaccharides.

The identity of these forms as glycosylated hCBG is confirmed by the observation that treatment with tunicamycin, an inhibitor of core glycosylation, results in disappearance of these species.

Time course of synthesis and secretion of hCBG in sf9 cells : To determine the efficiency and time course of secretion of hCBG from *sf9* cells, and to determine if there is significant protein degradation, a pulse-chase labelling experiment was performed. Infected cells were metabolically labeled for 1 h in presence of ^{35}S-methionine, and the labeled products were chased for increasing times in the presence of excess unlabeled methionine. At different time intervals, cells and medium were harvested and lysed, and hCBG was isolated by immunoprecipitation (Fig. 5). The precursor form of hCBG appears in the medium as early as 30 minutes of chase, however it appears to be degraded very quickly (Fig. 5A, lanes 3, 4 and 5). The glycosylated forms appear in the medium much more slowly, with a $t_{1/2}$ of 90 minutes (Fig. 5B, lanes 5 and 6). The summation of the total glycosylated hCBG of inside and outside shows that there is also some degradation process within the first 30 minutes of chase, but after that period the glycosylated forms are highly stable. At the end of 5 hours of chase, about 70% of the total pulse-labeled total glycosylated forms are secreted into the medium (Fig. 5B), and the glycosylated hCBG is the predominant form (lane 7).

These results indicate that glycosylated, signal cleaved hCBG is almost quantitavely secreted (Fig. 5B) from *sf9* cells. Also, glycosylated hCBG is more stable

Figure 4. **Specific Expression of hCBG in *sf9* Cells.** *Sf9* cells infected with the recombinant baculovirus AcMNPV-CBG were metabolically labeled with ^{35}S-methionine (100 μCi/ml) for 2h at 66h postinfection, in the absence (a, lanes 4-6; b, lane 2) or presence (b, lane 3) of tunicamycin (+TM, 5 μg/ml). Cell lysates (b, lanes 4-6) and medium (b,lanes 2-3) were subjected to immunoprecipitation. In lanes 5 and 6, immunoprecipitates were treated with Endoglycosidase H (+EH) and N-glycanase (+NG) prior to electrophoresis. *In vitro* translation products (a, lane 1-3; b, lane 1) correspond to those shown in Fig. 3 (mb = membrane). All immunoprecipitates were resolved on 12.5% SDS-PAGE.


a
in vitro
in vivo
1
2
3
4
5
6
69
46
30
Gly hCBG
pre hCBG
-mb
+mb
+mb +EH
-
+EH
+NG
cell lysate
b
ivt
in vivo
1
2
3
-mb
+TM
medium

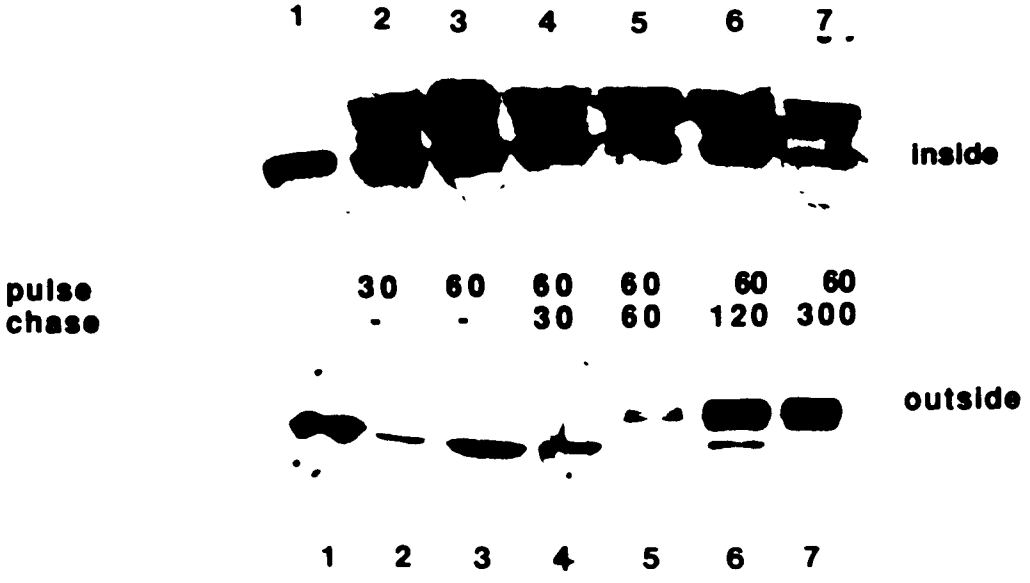
than the precursor form, as suggested by the rapid degradation of the latter.

High level production of hCBG in sf9 cells : Since the pulse-chase experiment suggested that recombinant virus infected *sf9* cells synthesize significant amounts of hCBG, I quantified the level of hCBG production. For this purpose, I developed a competitive immunoassay (Fig. 6). In this assay, a fixed amount of *in vitro* translated radiolabeled hCBG was allowed to bind to anti-hCBG antibody in the absence and presence of increasing amounts of either pure hCBG (Fig. 6a), human serum (Fig. 6b) or *sf9* cell lysate (derived from recombinant virus infected cells), (Fig. 6c). The amount of antibody added to each incubation was sufficient for 50% immunoprecipitation of the *in vitro* products.

There is a gradual loss of signal as an increasing amounts of either the cell lysate, pure hCBG, or human serum was added to the assay. The immuno-competition is linear in the range of 10ng to 50 ng of pure hCBG. By this method, the serum concentration of hCBG was found to be 22.5 μg/ml. The calculated amount of hCBG in cell lysate was 2.2μg per 10^6 cells (Fig. 6d). The total amount of hCBG present in medium derived from 10^6 cells was 1.2μg (Table I). This level of expression is comparable to that of other recombinant proteins expressed in the baculovirus/insect cell system. Also, the identical shape of the titration curves suggests that the antigenic determinants present in the hCBG derived from either human serum or *sf9* cells are very similar.

Biological activity and binding affinity of recombinant hCBG : To determine whether the recombinant hCBG is biologically active, a saturation ^{3}H-cortisol binding assay was performed. *Sf9* cells were infected at a high multiplicity of

Figure 5. **Time course of Synthesis and Secretion of hCBG in *sf9* cells.** *Sf9* cells infected with the recombinant baculovirus AcMNPV-CBG were pulse-labeled for 1h with ^{35}S-methionine (100 μCi/ml), and chased in the presence of 10 mM unlabeled methionine. hCBG was immunoprecipitated from cell lysates (top panel, A, lanes 2-7) and medium (bottom panel, A, lanes 2-7). Lane 1, top and bottom panel contains *in vitro*-translated precursor hCBG. Immunoprecipitates were resolved on SDS-PAGE and quantified by densitometry (B). ■ ,glycosylated hCBG; ▲, signal peptide containing hCBG.


1 2 3 4 5 6 7
inside
pulse 30 60 60 60 60 60
chase - - 30 60 120 300
outside
1 2 3 4 5 6 7

B.

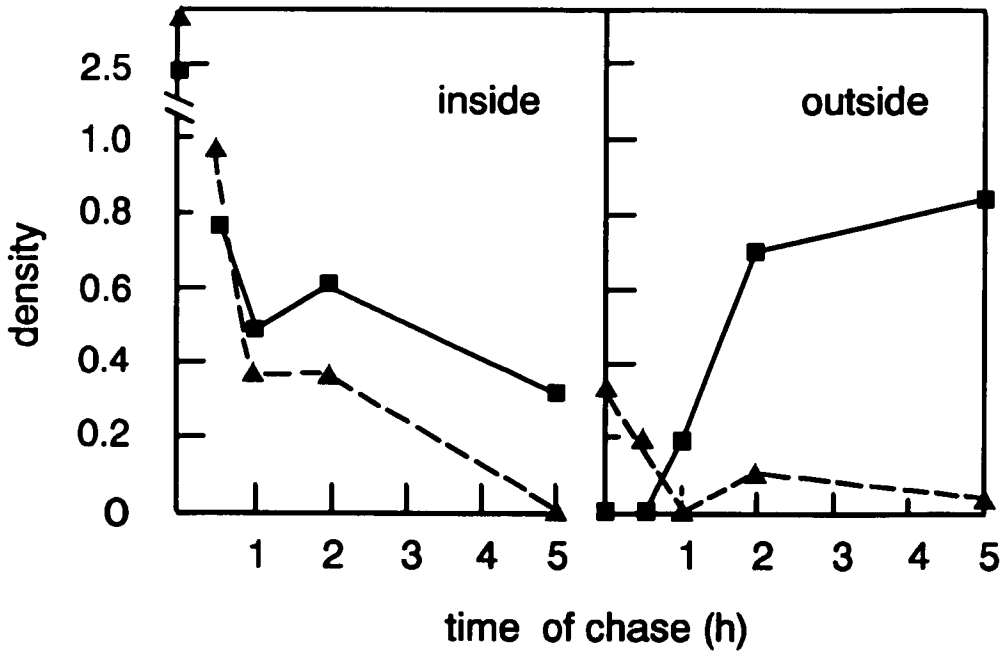
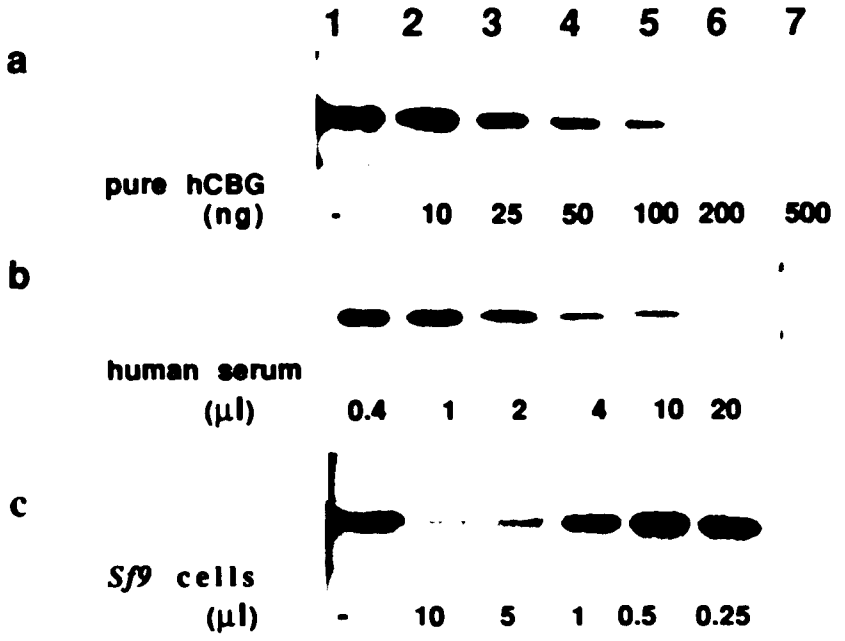
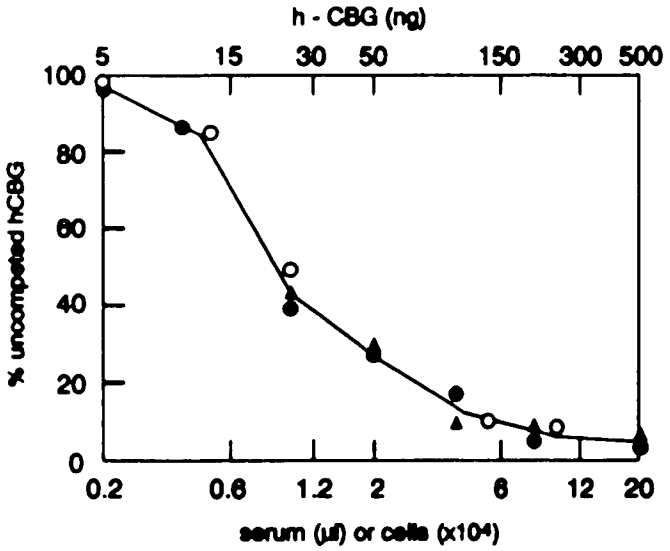

2.5
1.0
0.8
0.6
0.4
0.2
0
density
inside
outside
1
2
3
4
5
1
2
3
4
5
time of chase (h)

Figure 6. **High Level Production of hCBG in *sf9* Cells.** ^{35}S-methionine labeled *in vitro* translated products ($1X10^5$ cpm, as in Fig. 3, lane 1) were incubated with sufficient polyclonal anti-hCBG antiserum to achieve 50% immunoprecipitation in the presence of the indicated amounts of purified human serum hCBG (a), human serum (b), and *sf9* cell lysate (c). The immunoprecipitates were resolved on 12.5% SDS-PAGE and the amount of radiolabeled hCBG recovered in antigen-antibody complexes was quantified by densitometry (d) (Pure hCBG (●); human serum (▲); *sf9* cell lysate (○)).


1 2 3 4 5 6 7
a
pure hCBG
(ng) - 10 25 50 100 200 500
b
human serum
(µl) 0.4 1 2 4 10 20
c
Sf9 cells
(µl) - 10 5 1 0.5 0.25

d.


h - CBG (ng)
5
15
30
50
150
300
500
100
80
60
40
20
0
% uncompeted hCBG
0.2
0.6
1.2
2
6
12
20
serum (µl) or cells (x10⁴)

infection and were incubated with TNMFH (10% FBS) medium for 40 hours, after which cells were harvested and post-nuclear supernatants were prepared. Addition of increasing amounts of cytosol results in a linear increase in ^{3}H-cortisol binding (not shown). This binding is specific as it is fully inhibited by excess unlabeled cortisol; wild type virus infected cell cytosol exhibited only nonspecific binding.

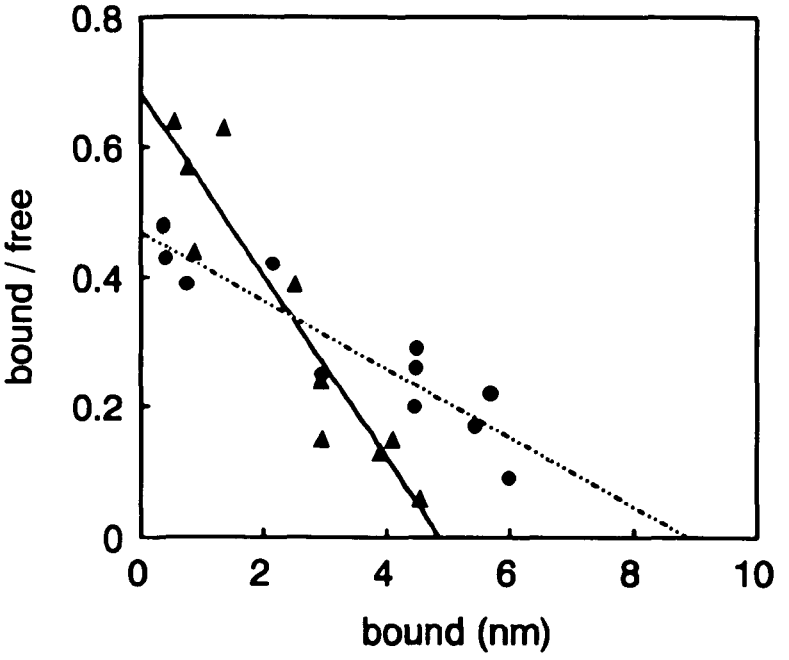
The affinity constant of *sf*9-derived hCBG for cortisol was determined by Scatchard analysis (Scatchard, 1949) using hCBG which had been secreted into the medium of recombinant virus-infected cells. The K_d value of expressed hCBG for cortisol was found to be 10^{-8}M (Fig. 7). This value is slightly lower than that of human serum-derived hCBG (10^{-9}M; Westphal, 1986). Ideally the estimated K_d approaches the true value when the concentration of the macromolecule is higher than K_d. But in the above measurement, the concentration of expressed hCBG in the serum free medium was lower. This resulted in low bound/free values with the low concentration range of ligands which might yield an underestimate of true K_d value.

Determination of relative steroid binding activity and comparison of specificities for different steroids: The relative steroid binding activities of the human-serum-derived hCBG and recombinant hCBG are compared in Table I. Total immunoreactive hCBG present in human serum, *sf*9 derived cell lysate or medium was quantified by immunoassay as described, and the total cortisol binding activity was determined by the saturation ^{3}H-cortisol binding method. The ratio of the total binding activity and total immunoreactivity is designated as relative binding activity (RBA). The results show that the *sf*9 medium derived hCBG and serum hCBG have

similar RBA values (0.8 and 1.08 respectively) whereas *sf9* cell-lysate derived hCBG has a very low RBA (0.05). We speculate that intracellular hCBG may represent a mixture of totally active and totally inactive species or molecules with partial activity. It has not been yet determined to what extent the intracellular hCBG is converted with time to active extracellular hCBG. It is possible that intracellular hCBG is mostly associated with a factor(s) which sequesters the steroid binding site and may dissociate from hCBG prior to secretion. Another possibility is that, during the early phase of biogenesis, there is proper folding of hCBG which is quantitaively secreted and is stable in the medium; at the later phase of viral infection, however, cytopathic effects may result in the accumulation of malfolded proteins. Possibly, synthesis of some component which is involved in the folding process in the secretory pathway may be inhibited, resulting in production of malfolded hCBG.

To investigate whether recombinant hCBG has identical affinities and specificities for different steroids as serum hCBG, a 50% saturation binding analysis was performed. In this experiment, the concentrations of steroids at which 20-80% inhibition of total binding is observed were used to calculate the K_d values of those steroids for hCBG. The following formula gives the K_d values: $K_p = K_d (1 + (I)/K_i)$ where K_d and K_p are the actual and apparent dissociation binding constant of recombinant hCBG for cortisol, and K_i is the dissociation binding constant for the inhibitors (I). These results are summarized in Table II. These results indicate that *Sf9*-derived recombinant hCBG not only has similar affinities for cortisol, but also has the identical rank order of steroid binding specificities.

Figure 7. **Binding Affinity of Recombinant hCBG.** At 66 h post-infection, *sf9* cells were incubated with serum-free medium for 24 h; the medium was then concentrated several fold with buffer exchange to achieve pH 7.4. A Scatchard analysis was performed using the ^{3}H-cortisol binding. Two sets of data points were analyzed by linear least squares, thus resulting in two straight lines. The average of the K_d values obtained from these two lines is 10^{-8} M.


0.8
0.6
0.4
0.2
0
bound / free
0
2
4
6
8
10
bound (nm)

D. Correlation of Structure and Function/ Role of Glycosylation in the Biogenesis of hCBG

To investigate the role of glycosylation in the biogenesis and stability of *sf9* derived hCBG, unglycosylated hCBG was produced as follows: recombinant virus infected *sf9* cells were treated with tunicamycin for 5 hours, after which the medium was changed to serum free collection medium containing tunicamycin. After a further 16 hours of incubation, the medium was collected, concentrated, and tested for specific ^{3}H-cortisol binding. In addition, this material was adsorbed to concanavalin A-Sepharose to remove residual glycosylated material. Immunoblot analysis was performed to visualize the presence of unglycosylated hCBG in this latter sample. A parallel experiment was carried out in which infected cells were pretreated with tunicamycin as above, and then radiolabeled with ^{35}S-labeled methionine for 16 hours. Radiolabeled cell lysate and medium fractions were then subjected to immunoprecipitation with anti-hCBG antibody, and the immunoprecipitates were resolved on SDS-PAGE.

Fig. 8a shows that tunicamycin effectively inhibits N-linked glycosylation (lane 2 and 4) and that the unglycosylated material is secreted in the medium, although at a reduced level. The presence of unglycosylated hCBG in the medium was further confirmed by immunoblot analysis of the concentrated samples (Fig. 8b, lanes 2 and 3). There was some residual glycosylated hCBG in the tunicamycin treated sample (Fig. 8b, lane 2) which was completely removed by concanavalin A-Sepharose chromatography (lane 3).

Table I: Determination of Relative Steroid Binding Activity (RBA)

Source of hCBG	immunoassay (pmol)	^{3}H cortisol binding (pmol)	RBA value
human serum (100 µL)	50	54	1.08
Sf9 cell lysate (2.5×10^6 cells)	113	6	0.05
Sf9 cell medium (2.5×10^6 cells)	60	48	0.80

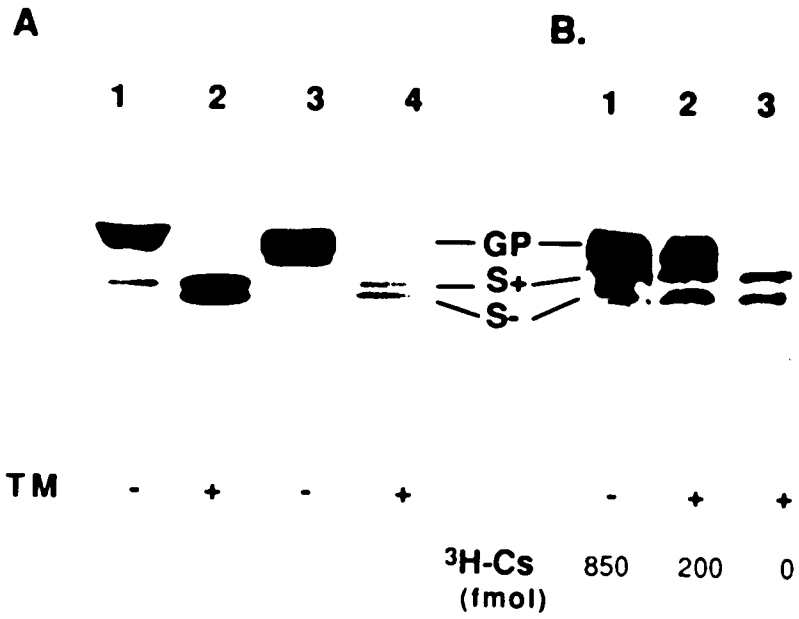
^{3}H Cortisol binding assay is linear between 25 and 250 fmol, and the standard error of binding is ± 16%. RBA is the ratio of the cortisol binding activity to the immunoreactivity of hCBG.

Table II: Comparison of Steroid Binding by Recombinant and Serum hCBG.

	human serum		*Sf9* cells	
steroid	K_a, M^{-1}	$K_a/K_a(Cs)$	K_a, M^{-1}	$K_a/K_a(Cs)$
Cortisol	2.6×10^8	1.0	1.8×10^8	1.0
Progesterone	4.3×10^7	0.17	2.2×10^7	0.12
Testosterone	1.8×10^6	0.007	1.0×10^6	0.006
Dexamethasone	5.5×10^4	0.0002	2.6×10^4	0.0001
Ecdysone	$< 1 \times 10^4$	—	$< 1 \times 10^4$	—

The association constants for cortisol binding to hCBG in human pregnancy serum and SF9 cell medium were determined by Scatchard analysis (Schiller & Petra, 1976). Association constants for the other steroids were determined by their competition at 50% saturation of binding. The error in the association constants is a factor of 2. $K_a(Cs)$ is the K_a for cortisol.

Figure 8. **Role of Glycosylation in the Biogenesis of hCBG.** Infected *sf9* cells were treated with or without tunicamycin (5 ug/ml) for 5 h at 40 h postinfection. In parallel cultures, media were then replaced with either methionine-free medium containing ^{35}S-methionine at 100 uCi/ml (A), or complete medium (B), lacking or containing tunicamycin (TM). In both cases, incubation was continued for an additional 16 h. (A) Labeled cells (lanes 1 and 2) and media (lanes 3 and 4) were harvested and subjected to immunoprecipitation with anti-hCBG antibodies, and the immunoprecipitates were resolved by SDS-PAGE. (B) Unlabeled culture media were harvested, concentrated, and incubated with concanavalin A-Sepharose to adsorb glycosylated polypeptides. The starting material (lanes 1 and 2) and the material that did not bind concanavalin A (lane 3) were resolved by SDS-PAGE and identified by immunoblotting. Saturation binding of ^{3}H-cortisol, measured in the same amount of material as was loaded on the gel, is indicated below the corresponding lanes (^{3}H-Cs).


A
B.
1
2
3
4
1
2
3
GP
S+
S-
TM
-
+
-
+
-
+
+
3H-Cs
(fmol)
850
200
0

The ^{3}H-cortisol binding analysis demonstrates that although the total tunicamycin-treated sample retains some residual activity, the purified unglycosylated hCBG has no binding activity at all (Fig. 8b, lanes 2 and 3, respectively). It should be noted that we measured ^{3}H-cortisol binding activity at pH 7.4. It could be argued that at higher pH, where binding is tighter, or with a different assay, partial activity may have been detected in the unglycosylated protein. However, at pH 7.4 cortisol binding is tight ($K_a \geq 10^8$) and the detection limit of the filter assay is 0.1 pmol of activity.

Although it has been shown by Michelson et al. (1982) that enzymatic deglycosylation of hCBG does not alter its steroid binding affinity, suggesting that the carbohydrate moieties are not directly involved in the steroid binding, the above results demonstrate that the co-translational N-glycosylation is important for hCBG to attain its biologically active conformation in the ER lumen. It appears that N-glycosylation alone is not the sole determinant of the active conformation, since hCBG derived from cell lysate has very low binding activity, in spite of the presence of carbohydrate on half of the intracellular material. Since the extracellular hCBG material, which is mostly glycosylated, is equally active as the native hCBG, it is possible that the intracellular hCBG undergoes further structural maturation during transport.

Summary

It has been shown by *in vitro* expression of hCBG cDNA in the RRL system that the precursor polypeptide contains an active signal peptide which allows membrane translocation. Signal peptide cleavage has been confirmed by the expected size of hCBG obtained after digestion with Endo H and N-glycanase. Furthermore, successful expression of biologically active hCBG in *sf9* cells has been achieved, and quantification of the level of expression shows that high amount of hCBG can be produced in this system for further physico-chemical studies. Cell biological characterization of recombinant hCBG has shown that these high mannose containing species have identical affinities and specificities for different steroids as that of native hCBG. These observations leads to the conclusion that subsequent processing of core-glycosylated hCBG in the Golgi compartment to complex carbohydrate containing species is not necessary for biological function. However, the present study brings evidence for the first time that the process of N-linked glycosylation is a crucial step in attainment of the steroid binding conformation of hCBG, although not the sole determinant.

CHAPTER II

Use of Site-Directed Mutagenesis and Chemical Modification to Investigate the Steroid Binding Site of hCBG/ Involvement of Cysteine Residue in the Binding Site

Introduction

Currently, site-directed mutagenesis is emerging as a useful tool to address issues such as the mechanism of protein folding, protein-protein interactions, and protein-ligand interactions. By introducing conservative amino acid changes, one can alter a local environment within a protein while maintaining the overall structure. Thus, this method allows us to investigate whether the side chain of a particular residue plays any crucial role in the structure and function of a protein. I have combined site-directed mutagenesis with chemical modification to investigate the possible role of cysteine residues in the steroid binding activity of hCBG.

Mature hCBG contains two cysteine residues, cysteine 60 and cysteine 228 (Hammond et al., 1987), and there is no disulfide bond in the final tertiary structure of hCBG. Affinity labelling studies using 6-β-Br-progesterone have suggested that a cysteine residue is present within the steroid binding site of hCBG (Khan and Rosner, 1977). However, it is not yet resolved whether the S-H group is involved in maintaining the binding site structure, or is directly involved in the interaction with the steroid ligand. Another question is, which of the two cysteine is in the binding site. The cDNA sequence of human, rat and rabbit CBG revealed that cys_{228} is conserved (Hammond et al., 1987; Smith and Hammond, 1989; Seralini et al., 1990) and is also within a conserved sequence. Thus, these observations led to the hypothesis that cys_{228} is present within the steroid binding site.

To investigate the role of cys_{228} in steroid binding , I have created two

mutant hCBGs in which the cys_{228} is replaced by serine and alanine (designated as hCBGS228 and hCBGA228, respectively). Creation of these two mutant proteins was intended to serve two purposes: 1) Observation of any change in the steroid binding properties of these mutants would directly identify cys_{228} as being involved in the binding process; 2) If the mutant proteins behave same as the wild type hCBG, the role of the remaining cysteine residue could be addressed by using sulfhydryl specific reagents.

Two mutagenic oligonucleotides were used in a two-step polymerase chain reaction along with 5' and 3' universal primers (oligo A and B respectively) to generate two PCR fragments which contained the desired serine and alanine codon in place of the cysteine codon (Fig. 9). In the first step, mutagenic oligo 3 or 4 was used with oligo A to prime the synthesis of an intermediate fragment containing the desired mutation. This fragment then served as the 5' primer in the second PCR, in conjunction with oligo B, to prime the synthesis of final PCR fragment. These final PCR fragments were digested with Sal 1 and BspE 1 and were reconstituted into the full length hCBG cDNA. They were then subjected to DNA sequencing to confirm the presence of the desired mutations. The full length mutated cDNAs were cloned in the baculoviral vector pVL1393 at the EcoRI site present at the downstream of polyhedrin promoter. Recombinant viruses were then isolated after transfection of *sf9* cells. The resulting recombinant viruses are designated as AcMNPV-CBGS228 and AcMNPV-CBGA228 which contained the serine and alanine mutations, respectively.

Results and Discussion

In vivo expression of mutant hCBGs : Expression and kinetics of mutant hCBGs secretion were monitored with a pulse-chase labelling experiment (Fig. 10 A,B). Infection with both mutant viruses results in synthesis of proteins which are recognized by anti-hCBG antibody and the distribution of molecular forms is similar to wild type hCBG, indicating that the posttranslational modifications of these mutants are similar to those of wild type hCBG. Furthermore, the secretion kinetics of the mutants are similar to that of wild type hCBG (Fig. 10B). After a 2h of chase, about 40% and 50% of the alanine and serine mutants, respectively, were secreted.

Biological activity of mutant hCBGs : To determine whether the replacement of cys_{228} with serine or alanine results in an alteration of steroid binding properties, Scatchard analysis was used to compare ^{3}H-cortisol binding of wild type and mutant hCBGs. The results (Fig. 11) show that, within the experimental error, all of these species exibit an identical K_d for cortisol ($1\text{-}2.5 \times 10^{-9}$ M at pH 8.0; these values are an order of magnitude higher in affinity than what was obtained at pH 7.4, consistent with what is reported in the literature (Westphal, 1986)). From these results it is concluded that sulfhydryl of cys_{228} is not involved in the steroid binding process, although it may still be present near the binding site.

Effect of sulfhydryl-specific reagents on the steroid binding activities of wild-type and mutant hCBGs : Mutation of cys_{228} of hCBG to serine or alanine does not change the steroid binding, although the literature suggests that a cysteine residue is involved in steroid binding. I therefore investigated the possible role of

Figure 9. **Strategy for Site-directed Mutagenesis by Polymerase Chain reaction (PCR)** . Step 1: The mutagenic primer (oligo 3 or 4) and the 5' primer (oligo A) are used in the first PCR to generate the intermediate fragment. Step 2: In the second PCR, the entire intermediate fragment is used as 5' primer along with the 3' primer (oligo B) to yield the final PCR fragment. Step 3: The final PCR fragment was digested with Sal I and BspE I to obtain the final fragment for cloning.

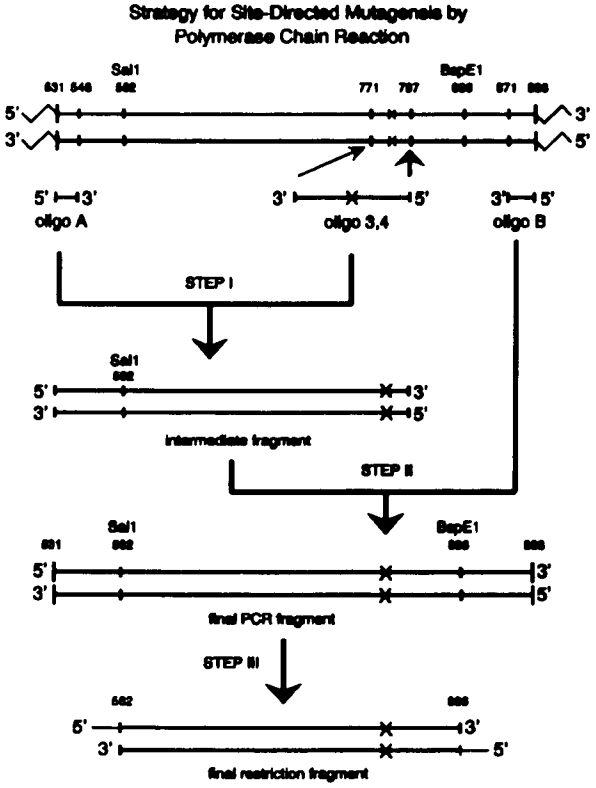
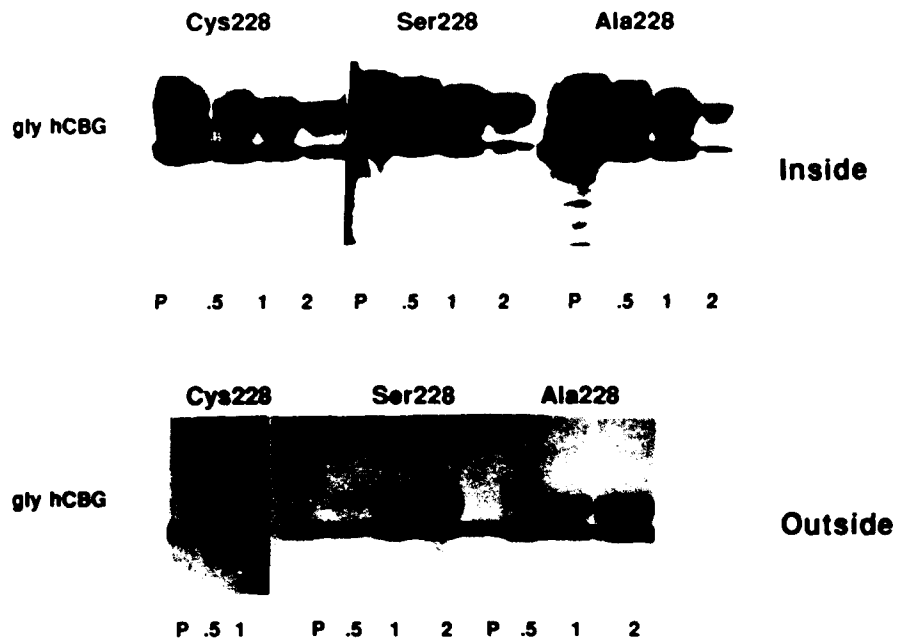

Strategy for Site-Directed Mutagenesis by
Polymerase Chain Reaction
Sal1
BspE1
631 648 662
771 787 888 871 886
5'
3'
3'
5'
5' 3'
oligo A
3' 5'
oligo 3,4
3' 5'
oligo B
STEP I
Sal1
662
5' 3'
3' 5'
intermediate fragment
STEP II
Sal1
BspE1
661 662 886 886
5' 3'
3' 5'
final PCR fragment
STEP III
662 886
5' 3'
3' 5'
final restriction fragment

Figure 10. **Comparison of Time Course of Synthesis and Secretion of Wild-type and Mutant hCBGs**. A: *Sf9* cells infected with recombinant baculovirus encoding wild-type (□,■; cys228) or mutant (○,●; Ser228) and (△,▲; Ala228) hCBGs were pulse-labeled for 1h (p) with ^{35}S-methionine (100 μCi/ml), and chased in the presence of 10mM unlabeled methionine for indicated times (0.5h, 1h, 2h). Cell lysates (A, Inside) and medium samples (A, Outside) were immunoprecipitated and resolved on 12.5% SDS-PAGE, and the total amounts of protein (glycosylated and precursor forms) were quantified by densitometry (B).


Cys228
Ser228
Ala228
gly hCBG
Inside
P .5 1 2 P .5 1 2 P .5 1 2
Cys228
Ser228
Ala228
gly hCBG
Outside
P .5 1 P .5 1 2 P .5 1 2

B.

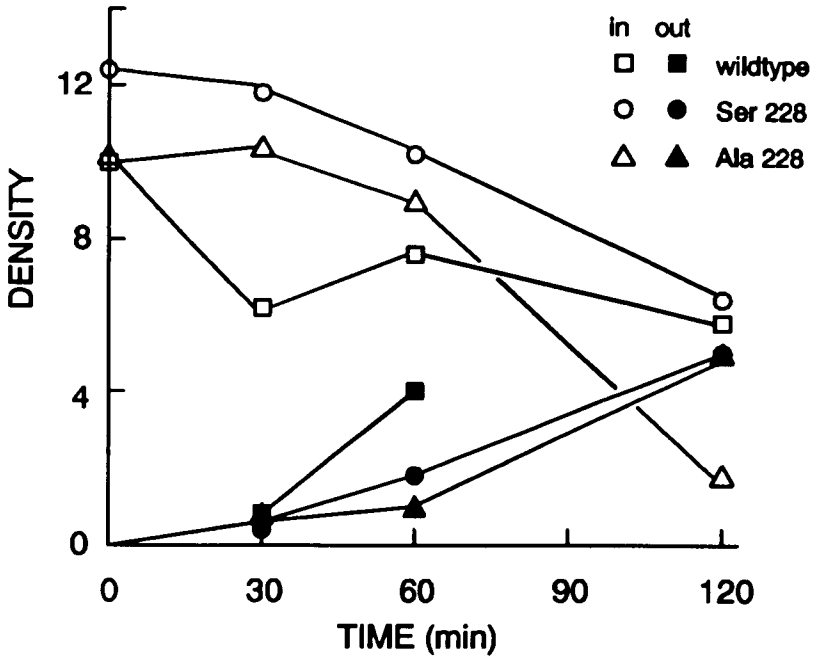
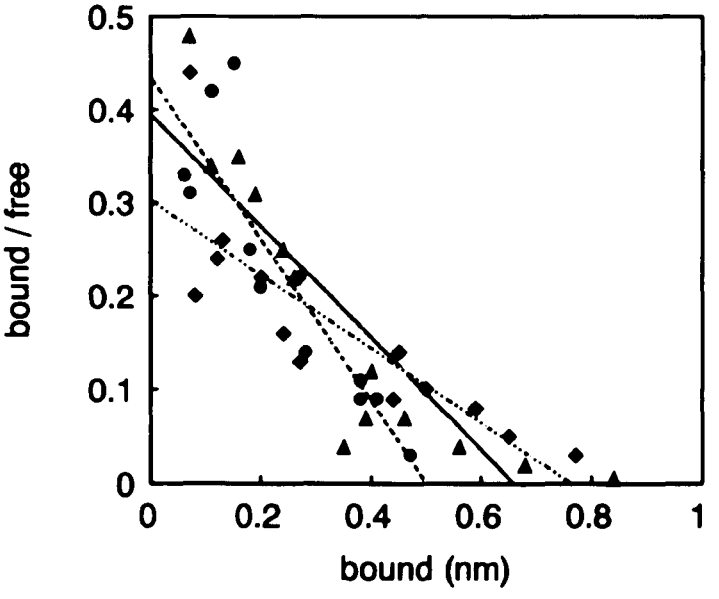

in out
wildtype
Ser 228
Ala 228
DENSITY
12
8
4
0
0
30
60
90
120
TIME (min)

Figure 11. **Binding Affinities of Mutant hCBGs.** At 66 h postinfection, *sf9* cells were incubated with serum-free medium for 24 h; the medium was then concentrated several fold with buffer exchange to achieve a pH 8.0. A Scatchard analysis was performed using ^{3}h-cortisol binding. (▲, —) wild-type; (●, - - -) Serine mutant; (♦, ⁻••⁻) Alanine mutant.


0.5
0.4
0.3
0.2
0.1
0
bound / free
0
0.2
0.4
0.6
0.8
1
bound (nm)

cys_{60} in steroid binding. Since each of the mutant hCBGs contains cys_{60} only, comparison of the sensitivities of these mutants towards S-H specific reagents with that of wild type hCBG, should address the possible involvement of this residue in the binding process.

To determine whether the wild type and mutant hCBGs have differential sensitivities towards the sulfhydryl-specific reagent N-ethylmaleimide (NEM, Roberts and Rouser, 1958), ^{3}H-cortisol binding activities of the wild type hCBG, hCBGS228 and hCBGA228 were measured after treatment with NEM (table III). Serum-free medium derived from infected *sf9* cells was concentrated several fold with buffer exchange so that the final pH was 8.0. Samples were then treated with 200 mM NEM for 30 minutes with or without prior treatment with 50 mM dithiothreitol (DTT). In the case of DTT pretreatment, DTT was removed before NEM treatment. After NEM treatment, excess reagents were removed by passing the reaction mixture through a spin column of Sephadex G-25. Cortisol binding assays were performed using these samples.

NEM treatment in the absence of DTT results in 64% and 44% inhibition of binding activity of the wild type hCBG and hCBGA228, respectively. Pretreatment of these proteins with DTT increased the inhibition to 83% and 92% respectively. The mutant hCBGS228 shows similar NEM sensitivity (data not shown). In all cases, deactivation occurs within 10 minutes incubation with NEM. These results demonstrate that either cys_{60} is directly involved in the steroid interaction, or that this S-H group is present in close proximity to the binding site.

Table III: Effect of Sulfhydryl Reagents on the Steroid-binding Activity of Wild-Type and Mutants hCBG

	Wild-Type		Ala 228		Ser 228	
treatment	cpm	residual activity	cpm	residual activity	cpm	residual activity
IAA:						
control	2730		2347		650	
100 mM	34	1%	110	5%	37	6%
NEM:						
control	4200		1794			
200 mM	1523	36%	1012	56%		
(pretreat with 50 mM DTT)						
control	1551		1012			
200 mM	259	17%	83	8%		
$Na_2S_4O_6$: (pretreat with 50 mM DTT)						
control	9490		4132		1110	
100 mM	198	2%	450	10%	301	27%

Percent inhibition (%I) with IAA, iodoacetamide; NEM; N-ethylmalemide; and $Na_2S_4O_6$ sodium tetrathionate. Cortisol binding activity was assessed by filter assay using ^3H cortisol. DTT-treated samples were passed through a second G-25 spin column to remove excess DTT before treatment with NEM resulting in some loss of protein; control experiments show that up to 10 mM DTT does not inhibit steroid binding under assay conditions (saturating steroid).

Furthermore, treatment of these samples with 100 mM iodoacetamide (IAA), in a similar protocol as above, completely abolished the cortisol binding activity of both wild-type and mutant hCBGs (Table III). Further kinetic analysis of this IAA deactivation showed that it occurs within 30 seconds of treatment. Furthermore, iodoacetamide deactivation of steroid binding property does not require DTT pretreatment. However, the process of deactivation is very sensitive to pH, and takes place only at pH ≈ 8.0. These results again indicate strongly the presence of a cysteine residue in the steroid binding site, although it can not be ruled out that a histidine residue may also be modified under these conditions, since IAA is also known to react with histidine (Lundblad and Noyes, 1988).

To substantiate that cysteine modification is responsible for inhibition of steroid binding, another S-H-specific reagent, sodium tetrathionate (Liu, 1967), was used at 100 mM in a similar experimental protocol. This reagent almost completely deactivates the cortisol binding activity of both wild-type and mutant hCBGs within five minutes of treatment, indicating again the presence of a S-H group in the steroid binding site (Table III).

Summary

It has been demonstrated that cys_{228} of hCBG is not involved in the steroid binding process. The results from chemical modification of wild type and mutant hCBGs with S-H specific reagents strongly indicate, instead, that cys_{60} is present in the steroid binding site. Furthermore, the requirement that DTT pretreatment of the mutant hCBGs lacking cys_{228} is necessary to achieve complete inhibition of steroid binding by NEM and sodium tetrathionate, provides the first direct evidence that cys_{60} is the accessible cysteine in hCBG. This requirement for DTT pretreatment also shows that cys_{60} is partially oxidized in the native as well as in the recombinant proteins.

DISCUSSION

The major goal of the current work was to understand the molecular structure of the steroid binding site of human corticosteroid binding globulin (hCBG). Since hCBG has very high affinity for steroids like cortisol and progesterone, and since it has a monomeric structure with moderate molecular mass, it constitutes a good model protein for studying the mechanism of protein folding during biogenesis. Specifically, I wished to investigate how the microenvironment of a protein dictates the steroid binding specificities for different related steroids. Furthermore, since hCBG is a glycoprotein, the question arises as to whether glycosylation has a role in determining its structure and function. I aimed to investigate whether the sugar moieties in this molecule are involved in the steroid binding process, and whether cotranslational glycosylation is essential for this molecule to attain an appropriate steroid-binding conformation.

To these ends, I have cloned the cDNA of hCBG and, for the first time, demonstrated that it indeed encodes authentic hCBG. This is indicated by its apparent molecular mass, by its recognition by antibody raised against human serum-hCBG, and by its steroid binding activity. Inclusion of microsomes (derived from RER) in the *in vitro* translation reaction (Fig. 3), demonstrated that the predicted signal sequence actually targets the molecule to the rough endoplasmic reticulum (RER) (Blobel and Dobberstein, 1975) and allows translocation of the nascent polypeptide. This is relevant, since the sequence of hCBG cDNA shows that both

the first and the second AUG codons are competent to serve as translational initiation sites. Interestingly, the second AUG is in a better context for initiation, according to the Kozak rules (Kozak, 1989). While initiation at the first AUG would produce a precursor polypeptide containing signal sequence, initiation at the second AUG would produce a polypeptide lacking that signal sequence, but initiating with the first residue of mature hCBG. By showing that the signal peptide cleavage occurs along with membrane translocation, I have confirmed that the first in-frame AUG is selected for initiation. In conclusion, I have shown that this cDNA is indeed the authentic sequence for hCBG and has a functional signal sequence.

To understand the relationship between structure and function in hCBG, it was necessary to establish a high level expression system, which was achieved using the baculovirus/insect cell system (Summers and Smith, 1987). High level expression of hCBG (1-2 ug from 10^6 cells) was shown to occur in *sf9* cells. In addition, it has been shown that hCBG is quantitatively secreted from *sf9* cells with a $t_{1/2}$ of 90 min. To determine the affinity of *sf9*-derived hCBG for cortisol, I have performed a Scatchard analysis of the binding data obtained under equilibrium binding conditions. I have found that the recombinant hCBG has cortisol binding affinity (Kd $\approx 10^{-8}$ at pH 7.4) similar to that of native hCBG. Also the steroid binding specificities of this molecule are identical to that of native hCBG. It binds cortisol and progesterone with very high affinity but binds testosterone with low affinity, and does not bind dexamethasone or ecdysone. These results further demonstrate the authenticity of recombinant hCBG.

This is the first reported expression of biologically active hCBG using recombinant DNA technology. Successful expression of hCBG in this heterologous system has therefore allowed me to address these following issues: (1) How does this polypeptide acquire its active conformation during passage through the secretory pathway? (2) Where in the secretory pathway does hCBG attain its steroid binding conformation? (3) What are the molecular determinants that confer high affinity and specificity for steroid binding?

Through these experiments, I have established the validity of the baculovirus/ insect cell system for producing high level recombinant hCBG. This system will further allow one to produce milligram amounts of protein for crystallographic studies. Also, biochemical analysis of structure and function of hCBG became possible. Furthermore, this system has the additional advantage in that it allows us to produce hCBG completely free of its ligand. This is important because, previous experience suggests that quantitative removal of ligand from hCBG is a difficult process.

Role of glycosylation in the biogenesis and function of hCBG

hCBG has six sites for N-linked glycosylation, of which five sites are actually modified (Perini et al., 1982). These sites remain conserved when one compares sequences from different species. Previously, Mickelson et al (1982) demonstrated that enzymatically deglycosylated hCBG retains its steroid binding capability, with a similar affinity for cortisol as the untreated molecule. However, the

question remains as to whether the process of N-glycosylation and subsequent processing to complex type carbohydrates are essential for hCBG to acquire its active conformation.

In general, glycosylation exerts diversified effects on structure and function of proteins. It has been found that some glycoproteins, such as fibronectin (Olden et al., 1982), thyroid stimulating hormone (Weintraub et al., 1983), and acetylcholine receptor (Prives and Olden, 1980), are rapidly degraded when produced in the presence of tunicamycin, an inhibitor of N-linked glycosylation (Takatsuki et al., 1971). It has been suggested that, since the en bloc transfer of the core $Glc_3Man_9GlcNAc_2$ oligosaccharide occurs cotranslationally, the added oligosaccharides may influence subsequent protein folding. Inhibition of N-glycosylation thus may result in exposure of some protease sensitive sites, which are otherwise sequestered in the fully glycosylated form.

Since many of the other glycoprotein sequences expressed in *sf9* cells have been shown to contain only high mannose oligosaccharides (Greenfield et al., 1988, Kuroda et al., 1986, Possee, 1986, Wojchowski et al.,1987), it was of interest to determine whether hCBG also has only high mannose oligosaccharides, and, if so, if that change in carbohydrate structures has any effect on function.

In human serum derived hCBG, the carbohydrate chains appear to be heterogeneous with respect to the fucose content and the position of glycosidic linkages (Westphal, 1986), causing native hCBG to appear as a diffuse band in SDS-PAGE. By contrast, the recombinant hCBG has discrete glycosylated forms,

suggesting that the carbohydrate moieties of *sf9*-hCBG are less heteregeneous than in human serum-hCBG. Notably, these carbohydrates are exclusively of the high mannose forms in both cellular and secreted materials, as shown by their sensitivity to digestion by endoglycosidase H. Since I have shown that the secreted material is capable of ligand binding, this suggests that addition of terminal sugars which normally occurs in human liver, is not required for biological activity.

I further investigated the role of glycosylation in the steroid binding process of hCBG. Unglycosylated hCBG was produced in these cells by treatment with tunicamycin. Tunicamycin treatment did not reduce the overall synthesis of hCBG, as evident from the amount of material present inside cells; however, it greatly reduced the secretion of hCBG. In a parallel experiment, in which radiolabelled hCBG was produced, it was found that there is some residual glycosylated species in the tunicamycin treated sample. To ensure complete removal of glycosylated hCBG, the sample was passed through concanavalin A-Sepharose (Con A). I have found that the hCBG which did not bind Con A lost steroid binding capacity, although it could be easily detected by immunoblotting (Fig. 8b).

Comparison of the results obtained from chemically deglycosylated hCBG (Michelson et al., 1982) to the behavior of the unglycosylated *sf9*-hCBG discussed in this work suggests that although the sugar moieties are not directly involved in the binding process, the cotranslational process of glycosylation is essential for folding of hCBG into a steroid binding conformation.

In case of hCBG, inhibition of N-glycosylation might have resulted in

some tertiary structure changes that do not affect stability *per se* but cause the polypeptide to be biologically inactive. The reduced level of secretion of hCBG by tunicamycin treated cells may be due to formation of heterooligomeric complexes with some cellular factors, as suggested by Jarvis et ail (1990) for other recombinant glycoproteins expressed in *sf9* cells.

Structural maturation

In order to determine whether the signal peptide containing- and the core-glycosylated hCBG obtained from *in vitro* translation has biological activity, I have used ^{3}-H cortisol binding assay (not shown). I have found within the detection limit of the filter assay, neither of these shows any binding activity. When compared to results of *in vivo* expression, this result indicates that signal peptide cleavage is necessary but not sufficient for acquisition of binding activity. It is evident that some other cellular factors are involved in the biogenesis of the fully functional hCBG.

I have also shown that the hCBG secreted in the extracellular medium is similar in its relative binding activity to native hCBG whereas intracellular hCBG exhibits very poor relative binding activity.

Attention has been focused recently, on a group of proetins called "chaperones" which are a subset of the heat shock protein (Hsp) family (Gething and Sambrook, 1992). An emerging concept is that these proteins interact with nascent polypeptides to prevent misfolding and intermolecular aggregations, and allow membrane translocation and proper folding. It has been shown with glucocorticoid

receptor, that in its untransformed state, it is associated with Hsp 90, and this association occurs soon after translation (Dalman et al., 1989). Furthermore, it has been demonstrated that this interaction is required for the steroid binding activity (Picard, et al., 1990). A similar situation may arise in case of hCBG. In other words, hCBG may be associated with secretory pathway chaperones such as heat shock proteins. The result of this association may be the sequestration of the steroid binding site, and release from this factor may occur prior to secretion.

Characterization of the steroid binding site by site directed mutagenesis and chemical modifications

Chemical modification studies on hCBG purified from human serum, suggested the involvement of a cysteine residue in the steroid binding site of hCBG (Westphal, 1986). Although the steroid binding domain of hCBG has not been delineated, when sequences from several species are compared, only one of the two cysteines, cys_{228}, is found to be in a highly conserved sequence (Hammond et al., 1987, Smith and Hammond, 1989, Seralini et al., 1990).

Several studies have been done previously to characterize the steroid binding site in human serum hCBG. Khan and Rosner (1977) identified a cysteine residue by affinity labeling using 6-β-Br progesterone. They found that the inhibition of cortisol binding by this steroid analog was time-dependent and irreversible. Initially, the analog could be competed with ^{3}H-cortisol. However, after 10 minutes, a covalent bond was formed and inhibition was complete. Based on thin layer

chromatography of the hydrolysis products of affinity-labeled hCBG, they further reported that a cysteine residue is covalently modified with this analog. Furthermore, they claimed that one of the two thiol groups is lost upon this modification, as tested with Ellman's reagent. From these results, they concluded that a cysteine residue is present in the steroid binding site of hCBG.

Le Gaillard and Dautrevaux (1977) also investigated the steroid binding site by affinity labeling. They made synthetic steroids in which the A and B rings were modified (^{14}C-6-α or 6-β-Br-acetoxy progesterone). They were unable to displace ^{3}H-cortisol from the binding site using this compounds. They concluded that the active site region surrounding the A and B ring of steroids is narrow and does not permit the entrance of a steroid analog if the A or B ring is substituted with a bulky group.

They also synthesized 11, 16 or 17 substituted steroids (11-α-Br-acetoxy progesterone, 16-α-Br-acetoxy-progesterone or 17-β-Br-acetyl-testosterone) to further investigate the effects of bulky substituents on the C and D rings. These steroid analogs could be used for affinity labeling of hCBG, and the results suggested that the region surrounding the C and D rings is larger. By acid hydrolysis of the affinity-labeled hCBG, they identified that a methionine and a histidine residue were modified. In a later work (1978), they were also able to amino-ethylate hCBG with ethylene imine and observed, on the basis of S-H titration, that prior binding of cortisol did not prevent modification. They also stated that aminoethylation of native hCBG does not destroy steroid binding activity, although they did not show any data

in support of that statement. Since they did not mention the concentration at which aminoethylation was performed, it is difficult to evaluate their conclusion.

I have probed the structure of steroid binding site of hCBG by use of mutant hCBGs expressed in insect cells. To address whether cys_{228} is involved in the steroid binding site, I have created two mutant hCBGs in which cys_{228} has been changed either to serine (hCBGS228) or to alanine (hCBGA228) by site-directed mutagenesis. Serine is a close analog of cysteine both chemically and sterically. If the cysteine S-H group is actually involved in hydrogen bonding with some part of the steroid molecule, I expected that interaction to be maintained in the serine mutant, since it retains that hydrogen bonding possibility. However, in the alanine mutant, I expected that this kind of interaction should be abolished since the side chain of alanine has a $-CH_3$ group instead of $-CH_2$-S-H as in cysteine; and thus the possibility of H-bonding has been eliminated. Also, the side chain of alanine is smaller than cysteine. These two mutants were expressed in the insect cell/baculovirus system in order to compare the properties of the wild type and mutant proteins.

The results from a pulse-chase labeling experiment show that the rate of synthesis and secretion of the wild type and mutant hCBGs are very similar. This result suggested that these mutant proteins are expressed normally in the insect cells. Their molecular forms are also identical with that of wild type hCBG, which further suggests that the post-translational modifications of these mutants are identical with that of wild type protein. Scatchard analysis of steroid-binding data obtained from the filter assay (Schiller and Petra, 1976), shows that both the mutant hCBGs have very

high affinity for cortisol and within the experimental error, these values are identical with that of wild type hCBG.

The result that hCBGS228 still binds steroid is not unexpected, as serine maintains a similar chemical environment as cysteine. The pH optimum of hCBG-cortisol binding is ≈ 8.0, which is the expected pKa of the cysteine S-H. This suggests that the $R\text{-}S^-$ form could be the interacting species at the binding site. However, I expected that if cys_{228} S-H is directly involved in steroid binding in the native hCBG, changing cysteine to serine should lower the affinity, since O-H of serine is a weaker nucleophile than the S-H group. Since hCBGS228 has identical affinity for cortisol, it is indicated that cys_{228} S-H might not be directly involved in steroid binding. Further observation that hCBGA228, in which the hydrogen bonding ability has been eliminated, is equally active, strongly supports the above conclusion.

Based on the binding activity of the mutant hCBGs, it can be hypothesised that cys_{60} rather than cys_{228} is the group involved in the steroid binding To test the role of cys_{60} in the steroid binding site, I have chemically modified the wild type and mutant hCBGs with S-H specific reagents N-ethyl maleimide (Roberts and Rouser, 1958), iodoacetamide (Lundblad and Noyes, 1988) and sodium tetrathionate (Liu, 1967). Both N-ethylmaleimide and iodoacetamide irreversibly modify S-H groups, whereas sodium tetrathionate is a reversible S-H specific reagent.

Le Gaillard and Dautrevaux (1978) reported that modification of the accessible S-H with ethylene imine did not induce any loss of binding activity in hCBG. Although the previous investigations pointed out that the buried cysteine

residue is present within the steroid binding crevice and is important for activity (Defaye et al., 1980; Le Gaillard et al., 1978), the present work provides evidence that the accessible cysteine (that was previously shown to be modified by sulfhydryl specific reagents upon reduction only) is within some proximity to the steroid binding site. After treatment with 200 mM N-ethylmaleimide (NEM), both the wild type and the mutants lost their binding activity. I have also found that when the proteins are not treated with DTT, only 44-64% inhibition of binding activity observed, whereas 83-92% inhibition occurs when DTT-treated proteins were used.

In addition, when the wild type and mutant hCBGs are treated with 100 mM iodoacetamide, there was complete inhibition of steroid binding activity (Table III). I also found that this inactivation is completely pH dependent (pH optimum ≈ 8.0, data not shown). This further indicates that a S-H group is actually modified, since pK of the cysteine S-H is ≈ 8.0). Furthermore, the observation that the kinetics of this inactivation are very rapid (30 seconds for 100% inactivation) strongly suggests that an S-H group, as opposed to a histidine, is the target for modification (the rate of histidine modification by IAA is very slow). Inactivation by iodoacetamide does not require prior reduction of wild type and mutant proteins.

The above results indicate that the accessible S-H, which is partially oxidized in the native protein, is the one which is modified by sulfhydryl reagents. The loss of steroid binding activity in mutant proteins (which lack cys_{228}), strongly suggests that either cys_{60} is present within the steroid binding site and /or is involved in the binding process directly, so that covalent modification of its S-H group with

a bulky compound sterically hinders steroid binding. The fact that prior reduction is not required to achieve complete inactivation by IAA may be explained by the high reactivity of iodoacetamide (since iodine is a good leaving group). Possibly, IAA may even react with a partially oxidized S-H group.

To confirm that a cysteine residue is modified in above experiments, I tested the effect of another reagent, sodium tetrathionate which has been successfully used previously in reversibly blocking S-H group (Liu, 1967). Sodium tetrathionate also completely destroys the activity of both wild type and mutant hCBGs. Complete loss of activity, however, is dependent upon prior reduction. This result confirmed that cys_{60} is the reactive group.

In conclusion, my thesis work has revealed important features of hCBG which are related to the steroid binding site as well as to the maturation of this polypeptide during biogenesis. First, I have shown that the cotranslational process of glycosylation *in vivo* is a crucial step in the protein folding pathway during the bigenesis of hCBG in ER lumen, although further processing of sugar moities to complex type in the Golgi apparatus is not necessary for acquisition of active conformation. Second, the binding results from hCBG mutants directly show that cys_{228} S-H is not involved in steroid binding; in addition, I presented the first evidence that cys_{60} may be involved in the binding process, or at least is in close proximity to the binding site.

Future Directions

1. To confirm whether cys_{60} is directly involved in steroid binding, construct and express hCBG mutants in which this residue is changed to serine or alanine.

2. Use fluorescent-tagged steroid, in conjunction with a mutant which contains only cys_{60}, to study the dynamic aspects of steroid binding.

3. Since tryptophan residues have been implicated as being in proximity to the steroid-binding site, construct mutants in which each of these four tryptophan residues has been deleted. This could identify which of these residues is involved, and provide further usuful hCBG variants for physico-chemical analysis.

4. determine if cortisol itself has any effect on the biogenesis of hCBG (in analogy to the effect of retinol on RBP). Compare insect cells which contain no cortisol or cortisol-binding proteins) with human hepatocyte cell lines. Investigate whether secretory pathway chaperone proteins bind hCBG and mediate structural maturation.

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